CLINICAL STUDIES OF BETA-THROMBOGLOBULIN

by

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Thesis presented for the degree of Master of Surgery

University of Edinburgh

1983

Abstract

Beta-thromboglobulin is a platelet specific protein of molecular weight 35,000, stored in the alpha granules and released during aggregation. Its precise function is unknown but it may act as a 'packing protein' in the alpha granules. Radioimmunoassays to measure it in plasma and urine have been developed.

Meticulous techniques of processing and sampling are necessary to prevent artefactual release. In healthy subjects the upper limit of the normal range is 80 ng/ml in plasma and 0.21 ng/ml in urine.

If the βTG concentration of the plasma is artificially elevated it is cleared exponentially with a plasma half life of about 80 mins. Only a tiny proportion of the infused dose appears in the urine.

Many drugs are known to affect platelet function but Aspirin, although it has a potent anti-aggregatory action, has no effect on the plasma βTG concentration. Heparin, however, may produce raised plasma βTG concentrations some hours after injection.

Only 65% of patients with venous thromboembolism had elevated βTG concentrations on the day of presentation. The degree of rise in concentration was related to the presence of pulmonary embolism but not to the duration of symptoms or the extent of the thrombus. However, 85% of patients with venous thromboembolism had elevated urinary βTG concentrations and so the urinary βTG concentration is a more valuable diagnostic test of venous thromboembolism than the plasma concentration.

Operation had a considerable effect on plasma βTG concentration which was most marked in patients undergoing vascular surgery. Many factors affected the plasma concentrations following operation but major infections produce the greatest rise. A small, but statistically insignificant rise occurred at the time of development of deep vein thrombosis diagnosed by the ^{125}I fibrinogen uptake test. The other factors affecting the plasma βTG concentration in the post operative period grossly limited its value in the diagnosis of post operative deep vein thrombosis. It was likewise of little value in the identification of graft thrombosis in those undergoing arterial reconstructions. The urinary βTG assay had similar disadvantages to the plasma assay in the post operative period.

The study on patients with atherosclerosis revealed that many had elevated plasma concentrations regardless of the clinical extent of the disease but most patients had normal urine concentrations.

The assessments of plasma and urinary βTG concentrations are a sensitive measure of platelet activity in experimental situations but the many factors which affect them and the relatively short plasma half life severely limit their clinical use.

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Acknowledgements

The work in this study was performed whilst I was employed as a Registrar in General Surgery at the Western General Hospital, Edinburgh, and as Senior Registrar in General Surgery at the Royal Infirmary of Edinburgh.

I acknowledge with gratitude the extensive help and advice of my supervisor, Mr C.V. Ruckley, who introduced me to the study of beta-thromboglobulin and gave me much encouragement.

This project was the work of a team and my role was confined to its clinical aspects. I was involved in the planning of the project from its inception and was responsible for the organisation of all its clinical aspects including the blood sampling and the ^{125}I fibrinogen uptake tests. I also performed much of the sampling and some of the ^{125}I fibrinogen uptake tests. At no stage was I involved in the processing or assaying of the specimens.

Mrs Jeanette Duncanson, a research nurse, performed the sampling and documentation of our post-operative study and Dr R. Cameron of the study of atherosclerosis. I am also grateful to Dr John Simpson, Department of Medical Physics, Western General Hospital, whose staff performed most of the ^{125}I fibrinogen uptake tests.

The initial preparation of the specimens was carried out by technicians in the Department of Haematology under the direction of Dr Norman Allan at the Western General Hospital and in the Department of Blood Transfusion under the direction of Dr John Cash at the Royal Infirmary. Dr Cash also provided continual support and

advice throughout the project which was appreciated.

The radioimmunoassays were performed in the Medical Research Council's Radioimmunoassay Laboratory, Forrest Road, Edinburgh, by Dr A.E. Bolton and Dr Joan Dawes under the direction of Dr W.M. Hunter.

I am indebted to the Surgeons and Physicians of the Western General Hospital, Edinburgh, and the Royal Infirmary of Edinburgh who allowed me to investigate their patients, and also to all my colleagues - nurses, students and doctors - who contributed blood samples for the study.

Finally, I thank the Radiochemical Centre at Amersham for their financial assistance which funded Mrs Duncanson and the ^{125}I fibrinogen uptake tests.

Publications

Some of the work in this thesis has already been published in the following:-

1. Beta-thromboglobulin in venous thromboembolism.
   In: *New Trends in Venous Diseases,* pp. 136-142, 1978.
   A. Kappert, Hans Huber, Berne.

2. Beta-thromboglobulin and deep vein thrombosis.
   Smith, R.C., Ruckley, C.V., Duncanson, J., Allan, N.C., Dawes, J., Hunter, W.M., Pepper, D.S. and Cash, J.D.
   Thrombosis and Haemostasis, 39, 338-345, 1978.

3. The release, distribution and clearance of human beta-thromboglobulin and platelet factor 4.
   Dawes, J., Smith, R.C. and Pepper, D.S. *Thrombosis Research,* 12, 851-861, 1978.

4. Beta-thromboglobulin and deep vein thrombosis in surgical patients.
   Smith, R.C., Duncanson, J., Allan, N.C., Dawes, J., Pepper, D.S., Cash, J.D. and Ruckley, C.V. *Thrombosis and Haemostasis,* 38, 166, 1977.
   (Proceedings of the VIth International Congress of Thrombosis and Haemostasis) Abstract.

5. Beta-thromboglobulin clearance.
   Dawes, J., Smith, R.C., Hunter, W.M., Duncanson, J., Ruckley, C.V., Allan, N.C., Pepper, D.S. and Cash, J.D.
   Thrombosis and Haemostasis, 38, 314, 1977. (Proceedings of the VIth International Congress of Thrombosis and Haemostasis) Abstract.

6. **Urinary beta-thromboglobulin and venous thromboembolism.**
Smith, R.C., Dawes, J. and Ruckley, C.V. *British Journal of Surgery,* **66, 368, 1979. (Proceedings of the Surgical Research Society) Abstract.**

7. **The value of urinary beta-thromboglobulin in clinical situations.**
Dawes, J., Smith, R.C., Bosey, D. and Aronstam, D. *Thrombosis and Haemostasis,* **42, 147, 1979. (Proceedings of the VIIth International Congress of Thrombosis and Haemostasis) Abstract.**

Presentations

Aspects of this thesis have been presented at the following major meetings:-

1. International Meeting on Venous Diseases, Nyon, Switzerland, 1976.
2. VIth International Congress of Thrombosis and Haemostasis, Philadelphia, USA, 1977.
3. VIIth International Congress of Thrombosis and Haemostasis, Paris, France, 1978.
4. Surgical Research Society, London, 1979.

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List of Abbreviations used in the Text

ADP	adenine diphosphate
βTG	beta-thromboglobulin
DVT	deep venous thrombosis
E	ethylene diamine tetra-acetate
FDP	fibrin/fibrinogen degradation products
FPA	fibrinopeptide A
FPB	fibrinopeptide B
GS	general surgical
HNA	heparin-neutralising activity
5HT	5-hydroxy tryptamine [serotonin]
HTCT	heparin-thrombin clotting time
$^{125}_{IFT}$	^{125}iodine fibrinogen uptake tests
MI	myocardial infarction
MRC	Medical Research Council
P	prostaglandin E_1
PDGF	platelet derived growth factor
PE	pulmonary embolism
PF_3	platelet factor 3
PF_4	platelet factor 4
PGI_2	prostacyclin
PVD	peripheral vascular disease
RIA	radioimmunoassay
SD	standard deviation
T	theophylline
VPEF	vascular permeability enhancing factor

INTRODUCTION

THE PROBLEM OF THROMBOEMBOLIC DISEASE

In 1846 Rudolph Virchow[189] first described venous thromboembolism and in 1856[190] published his theory of the origin of intravascular thrombosis which became known as 'Virchow's Triad'. He attributed the development of a thrombus to one or more of three factors, namely, damage to the vessel wall, hypercoagulability of the blood and retarded blood flow. This theory formed the basis of most of the subsequent research on the aetiology of intravascular thrombosis. Bizzozero in 1882[17] demonstrated that injury to a vein wall resulted in the adherence of platelets to the damaged area with superimposed aggregation and Hayem,[75] in the same year, described the haemostatic platelet plugs that developed at the sites of puncture wounds in veins. Following the excellent description of the morphology of intravascular thrombus by William Welch,[197] it has been apparent that thrombosis is merely a pathological extension of the normal haemostatic mechanism. The development of phlebography and arteriography[12,47,48,126] led to the study of the pathological anatomy of thrombosis during life and, with increasingly sophisticated techniques, very precise visualisation of thrombi has become possible.

The incidence of diseases associated with intravascular thrombosis appears to be increasing. Deep vein thrombosis following surgery became more frequently recognised in the early years of this century but it probably occurred less often than it

does today. If one looks at the reported incidence of death from pulmonary embolism - a late and serious complication of venous thrombosis - there was a nine-fold increase in the 34 years between 1945 and 1978 (Fig. 1).[161] The rise in females was slightly higher (x 10) than in males (x 7) and it is unlikely that this increase was due purely to a greater awareness of the disease. The increasing incidence of thrombotic disease was not confined to the venous circulation. Death from coronary artery disease, a very much commoner cause of death than pulmonary embolism, increased four-fold in the same 34 year period (Fig. 1).[161]

The seriousness of these trends has stimulated further research into the mechanisms of thrombosis and the diagnosis of thrombotic disease.

Welch[197] showed that thrombus consists of fibrin, platelets, erythrocytes and leukocytes in varying proportions and the following section summarises the current understanding of the mechanisms of formation of this complex structure.

THE MECHANISM OF THROMBOSIS

There are two principal processes involved in the formation of a thrombus - the fibrin generation system and the adhesion and aggregation of platelets - and they are very closely inter-related.

Endothelial damage exposing subendothelial collagen is an important precipitating factor in at least some forms of intravascular thrombosis[176] and is well demonstrated in the arterial circulation where extensive thromboses occur in relation to

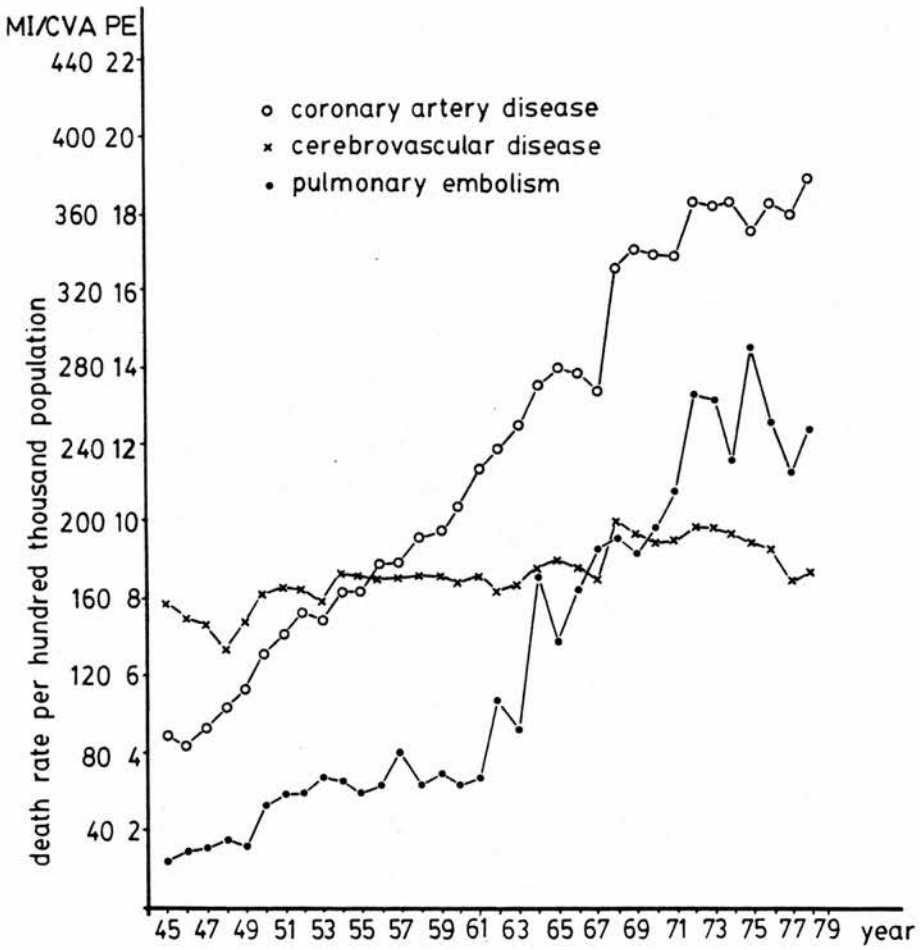

Fig 1: Mortality from coronary artery disease, cerebrovascular disease and pulmonary embolism, 1945-1978, in Scotland.
(Registrar General's Annual Reports for Scotland)

atherosclerotic endothelial damage. In venous thrombosis, however, it is often difficult to demonstrate changes in endothelial cells and it has been postulated that in this situation thrombosis may occur in the absence of endothelial damage.[192] Stasis leading to the activation of coagulation factors and relative hypoxia in the valve cusps may be responsible. However, damage to the endothelium may be present but only recognisable at an electron microscopic level, as Stewart *et al.*,[179] using scanning electron microscopy, have demonstrated areas of endothelial damage associated with leucocytes in a canine venous stasis model. They describe the attachment of thrombus by fibrin strands to leucocytes which have migrated through the endothelium.

The earliest stage of thrombus formation in response to endothelial damage is the adhesion of platelets to the exposed collagen[6] which is a potent stimulator of adhesion and aggregation. Endothelial damage also results in the activation of factor XII leading to the production of thrombin which will enhance platelet adhesion.[198] Factor VIII related antigen may also be involved.[196]

The adhesion and/or aggregation of platelets results in the platelet release reaction.[80] This involves the release from the platelet of the contents of the storage granules by a process similar to the secretion of the adrenal medullary hormones. These contents include adenosine diphosphate (ADP), serotonin (5HT), fibrinogen, platelet factor (PF_4), betathromboglobulin (βTG), platelet derived growth factor (PDGF) and vascular permeability enhancing factor (VPEF). The release of ADP results in the further aggregation of platelets to those already adherent.

The coagulation cascade, originating with factor XII activation by collagen, proceeds simultaneously and results in the production of thrombin which in turn converts fibrinogen to fibrin. Further stimulus to the production of thrombin is provided by PF_3, a membrane phospholipid released during platelet aggregation which catalyses the activation of factor X.[90] The released PF_4 will help by neutralising any heparin-like activity present. Fibrinogen is also released from platelets and this seems to be chemically distinct from the plasma fibrinogen.[86] Although it is present in very small amounts relative to plasma fibrinogen, it will increase the local concentration around the platelets producing added substrate for the thrombin.[199] The thrombin splits fibrinopeptides A & B (FPA & FPB) from fibrinogen to form fibrin monomer which then polymerises under the influence of factor XIII to produce the stable fibrin polymer.

The fibrinolytic system is stimulated at the same time as the fibrin generation system. Plasminogen, the inactive precursor of plasmin, is incorporated in the thrombus as it forms. Thrombin and intravascular fibrin stimulate the release of plasminogen activator from the vessel wall which converts plasminogen to plasmin and which in turn lyses fibrin with the production of fibrin degradation products (FDP).[3] As well as lysing the fibrin in the thrombus a mild plasma proteolytic action is generated which will produce some degradation of circulating plasma fibrinogen.

Thrombus is, therefore, a dynamic structure with fibrin generation and lysis occurring at the same time. One or other may predominate at different stages in the life of a thrombus.

This enormously complex biochemical process results in the release of many substances into the circulation which may be potential indicators of thrombotic disease. It would also be useful if some method of differentiating between venous and arterial thrombosis could be identified. However, the content of both types of thrombus is remarkably similar. Olson *et al.*[150] showed that in experimental conditions arterial thrombi contained 68 times the platelet content of blood, slightly less red cells than blood and twice the fibrin equivalent of plasma fibrinogen. Venous thrombi had similar fibrin and red cell contents but only 28 times the platelet content of blood. The assessments of these experimental thrombi give a somewhat artificial impression of their content in that thrombi in the venous circulation can have a very variable composition in different parts of the same thrombus. A good example of this is coralline thrombus with its alternating layers of platelet rich and platelet poor thrombus.[70] Therefore, at any point during the formation of such a thrombus there may be predominantly platelet or fibrin deposition and this may alter the types of substances detectable in the circulation.

THE PREDICTION AND DIAGNOSIS OF INTRAVASCULAR THROMBOSIS

Are diagnostic tests of thrombosis necessary? In the venous circulation, Kakkar[94] has demonstrated that clinical diagnosis of calf vein thrombosis is unreliable. Fifty per cent of those with evidence of thrombus on an ^{125}I fibrinogen uptake test had no symptoms or signs and 30% of those with symptoms or signs of calf DVT had normal deep veins on phlebography. However, in iliofemoral

thrombosis clinical accuracy over 80% if possible. Does it matter if one fails to detect these apparently trivial calf thrombi? The majority of these thrombi lyse spontaneously but about a quarter of them will propagate above the knee and these may give rise to major pulmonary embolism. Early diagnosis and treatment can prevent this serious complication. Even the thrombi that remain confined to the calf are not without problems, as Browse and Clemenson[22] have shown that many cause longstanding leg pain and swelling.

Arterial disease is also common in the UK as evidenced by the increasing incidence of death from myocardial infarction (Fig. 1).[161] Many of these deaths occur suddenly in people without previous symptoms or signs of arterial disease. Clinical examination may demonstrate major lesions of the mainstream arterial supply to the limbs and in the neck but tells us little about disease of smaller vessels such as those in the heart or brain. A simple method of detecting minor thrombotic disease in its early stages could possibly lead to earlier treatment with, hopefully, the prevention of major complications.

There is, therefore, a pressing need for the development of a satisfactory technique of detecting early intravascular thrombosis. A satisfactory test is one which reliably differentiates those with thrombosis from those without (i.e. it is sensitive and specific). Ideally it should also be non-invasive and harmless, cause no discomfort, able to be frequently repeated and inexpensive.

The currently available diagnostic tests are based on two different principles. The first type detects physiological changes

produced by the alterations in blood flow which result from intravascular thrombosis. Examples of these are the detection of alterations in blood flow by ultrasound velocity meters[173,180,206] or by plethysmography;[45,131,200] detection of altered tissue perfusion by isotope clearance,[174] thermography,[14] or by reduced arterial pressure;[174] or by the imaging of the thrombus by phlebography or arteriography.[12,47,48,126,171]

The most satisfactory delineation of the thrombus is provided by angiological techniques but these investigations have a small incidence of serious complications; they are unpleasant for the patient and are expensive. They are, therefore, unsuitable for screening. The tests which detect changes in blood flow are simpler, cheaper and non-invasive but less sensitive and specific than angiography, and fail to differentiate between new and old thrombus.

I do not wish to pursue this aspect of diagnostic techniques but will discuss further the second type of investigation which has attempted to use biochemical changes as indicators of thrombus formation.

These investigations may be divided into predictive tests (which identify individuals at particular risk of developing thrombotic disease) and diagnostic tests (which diagnose the presence of thrombus).

PREDICTIVE TESTS OF THROMBOSIS

Dawbarn's paper in 1928[39] is perhaps the earliest attempt to correlate haematological change with thrombosis. He demonstrated a rise in platelet count following surgery and parturition and suggested that it was a precipitating factor in the development of deep vein thrombosis. The extent of the rise was not related to patient age but was related to the extent of surgery. Payling-Wright[153] showed that, as well as a rise in platelet count following surgery and parturition, there was an increase in adhesiveness of the platelets. However, in 1969, Negus *et al.*[138] who used Wright's rotating bulb method of assessing platelet adhesiveness,[205] were unable to demonstrate any difference in adhesiveness before or immediately after surgery between those who developed DVT and those who did not. Becker,[9] however, using a different method (platelet retention in a glass bead column) showed that there was a significant relationship between the development of post-operative DVT and the platelet adhesiveness measured immediately after operation. In patients with recurrent venous thromboembolic disease, Steele *et al.*[178] were able to demonstrate a shortened platelet survival in 18 out of 28 patients but increased adhesiveness in only 8. Hirsch and McBride[77] also showed increased platelet retention in a glass bead column in these patients.

Whole blood viscosity may be related to thrombogenesis. Several factors contribute to viscosity but the most important appear to be haematocrit and the fibrinogen content.[53] Dormandy[46] showed a significant relationship between pre-operative whole blood viscosity (haematocrit corrected) and the development of DVT. The

patients who developed DVT had viscosities 29% higher than those who did not but the specificity of the test was poor.

The fibrinolytic system has been the subject of a great deal of research in the last 25 years and there is now good evidence that the fibrinolytic activity of the blood is derived from the release of plasminogen activators by the endothelium of small blood vessels.[3] Venous occlusion stimulates the release of vessel wall activator and this is the basis of a test of fibrinolytic activity. The resultant fibrinolytic activity can be measured by either euglobulin lysis time[191] or the dilute blood clot lysis time.[58] Pandolfi *et al.*[152] have also described a technique for assessing vessel wall fibrinolytic activity in vein biopsies which is a modification of the original fibrin plate method of Todd.[183] Using these techniques Mansfield[115] was able to show a significant relationship between post-operative DVT and reduced fibrinolytic activity in the blood on the first day after surgery but Becker[10] could not confirm this. In patients with recurrent idiopathic venous thrombosis, Isacson and Nilsson[85] showed defective fibrinolysis in both blood and vein wall and Jarrett *et al.*[87] were able to reverse the abnormalities with the fibrinolytic enhancer, Stanozolol. Browse *et al.*[24] reported similar findings in patients with recurrent superficial thrombophlebitis and venous liposclerosis.

The coagulation system has also been investigated for potential predictive value. Patients with a high kaolin activated partial thromboplastin time (indicating a degree of inhibition of thrombin generation) before operation appear to have a low risk of post-operative DVT whereas those with a high level of plasmin

inhibitor activity on the day after surgery have a high risk.[64]

In 1965 Egeberg[51] described a thrombophilic family who had an inherited deficiency of antithrombin III (which inhibits thrombin and activated factor X) and since then several other such thrombophilic families have been identified in Europe and North America. Antithrombin III levels fall following surgery but Åberg[1] was unable to correlate this with post-operative DVT. However, Okuno and Crockatt[148] were able to demonstrate reduced antithrombin III levels in 67% of patients with a recent DVT (within 4 months).

Similar studies have been performed on patients with arterial disease, with most interest being in the investigation of platelet function. An increased aggregability of platelets in response to ADP and collagen has been demonstrated in atherosclerotic patients,[133,149,194] and Abrahamsen[2] was able to show that they had abnormal platelet survivals. He also showed shortened survival in patients with acute myocardial infarction with return to normal on clinical recovery, a finding confirmed by Sano[170] who found similar changes in acute stroke patients. On young acute stroke patients Sharma[172] demonstrated increased platelet adhesiveness as well as increased plasma fibrinogen concentration.

Atherosclerotic patients have numerous areas of endothelial damage which may have an effect on both the platelets and the coagulation system. The increased platelet adhesiveness detected in the patients with acute stroke or myocardial infarction may be secondary to a recent thrombus rather than the cause of that thrombus.

Reduced platelet survival is found in patients with prosthetic heart valves and this seems to correlate with the incidence of arterial thromboembolism.[195] Finally, in vascular surgery, increased platelet adhesiveness has been associated with premature graft occlusion.[55,72]

These tests are concerned with the identification of populations at risk from thrombotic disease and are of little value in the diagnosis of acute or established thrombus.

DIAGNOSTIC TESTS OF THROMBOSIS

Diagnostic tests which assess the biochemical changes associated with acute thrombosis are of two types. The first type measures the incorporation of certain materials into the thrombus whereas the second type is dependent on the detection of substances released from or by the thrombus.

As shown earlier, the formation of a thrombus involves the deposition of platelets and fibrin in concentrations greater than in the circulating blood. Plasminogen and plasminogen activators are also incorporated. By labelling any of these substances with radioisotopes the label is incorporated into the developing thrombus and the thrombus can be detected by an area of increased radio-activity. The most widely used of these tests, the ^{125}I fibrinogen uptake test (^{125}IFT), utilises ^{125}I fibrinogen[7,21,52,137] and this has become the standard technique for diagnosing DVT in the major trials of prophylactic agents. It is relatively cheap, safe, simple and accurate but does have certain disadvantages. The ^{125}I

fibrinogen must be present in the circulation as the thrombus forms or propagates and, therefore, is of little value in diagnosing established non-propagating thrombi. It is also of little value in diagnosing thrombus above the inguinal ligament as the background radiation from the pelvic veins and the bladder obscures the radioactivity in iliac thrombus. Other substances have also been tried such as ^{131}I plasmin,[151] ^{99m}Tc urokinase[121] and ^{99m}Tc streptokinase.[99] Although these techniques have certain advantages over the ^{125}IFT, such as better delineation of established thrombus, their difficulties of manufacture, limited effective isotope half life or their unreliability have prevented their widespread acceptance. A more promising recent development has been the use of ^{111}In-labelled platelets.[59,100] The diagnostic accuracy of the test appears to be good when compared to phlebography and it has the advantage over the ^{125}IFT in that there appears to be a continual deposition of platelets even on established thrombus. Another potentially valuable aspect of the use of labelled platelets is that they are deposited on areas of endothelial damage and thus may be of some value in the study of arterial disease.

The second type of test - detecting changes in the circulating blood produced by thrombus formation - has been widely investigated.

Attempts to detect elevated levels of activated clotting factors in the circulating blood of patients with thrombosis are unlikely to be successful as they are very rapidly cleared from the circulation,[176] probably by the reticulo-endothelial system in the liver and spleen.[43]

The end result of the coagulation cascade is the conversion of fibrinogen to fibrin. Most of the fibrin then polymerises but some escapes into the circulation as soluble fibrin monomer complexes and can be detected in the plasma. Gurewich and Hutchinson[68] reported on the use of a serial dilution protamine sulphate test to detect soluble fibrin monomer complexes in the diagnosis of DVT. The test was commonly positive in those with positive venograms but rarely so in those with negative venograms. However, Gallus *et al.*,[64] who used the same test and the ethanol gelation test, were unable to show any useful correlation between these tests and the ^{125}IFT.

A chromatrographic technique for detecting fibrinogen/fibrin complexes in the plasma was developed by Fletcher *et al.*[63] and they showed that hypercoagulable patients tended to have lower elution volumes than normal patients.

During the formation of fibrin, thrombin cleaves two peptides from fibrinogen, and a radioimmunoassay has been developed to detect the first of these - fibrinopeptide A (FPA).[141,142] Nossel[142] has shown that FPA has a half life in the circulation of only $3\frac{1}{2}$ minutes and may be a marker of active fibrin deposition. High concentrations were detected in patients with DVT, PE, arterial thrombosis and myocardial infarction but he also found elevated concentrations in meningitis, thrombocytopenia and atherosclerosis. Other authors have also found elevated FPA concentrations in DVT and PE[155] and in patients with myocardial infarction.[89] There was some correlation between the FPA concentration and the size of the infarct. High concentrations of FPA have also been identified in

patients with metastatic carcinoma[156] and Davies *et al.*[38] have shown elevated concentrations following cardiopulmonary bypass. The FPA concentration can be used as an indicator of the adequacy of anticoagulant therapy in patients with thromboembolism[155] but, if the patient has a pulmonary infarct or a pleural effusion, the FPA concentration remained permanently elevated despite adequate anticoagulation. Nossel[142] also demonstrated the ability of heparin to reduce the FPA concentration but noted that FPA generation was never completely prevented which suggests that either FPA is formed somewhere out of the sphere of influence of heparin or that there is another mechanism for FPA generation not dependent on thrombin. This may explain the elevated concentrations detected in other conditions such as metastatic carcinoma and obviously limits the specificity of the test for thromboembolic disease.

With the activation of the fibrinolytic system fibrin and fibrinogen are broken down into smaller molecules with the generic name of Fibrin/Fibrinogen Degradation Products (FDPs). Patients with actively lysing thrombus are likely to have elevated concentrations of FDPs. These breakdown products were first described by Nussenzweig in 1960[144] and since then they have been extensively studied in many disease states. In 1969 Cash *et al.*[29] showed striking elevations of FDP in patients with suspected pulmonary embolism and this was confirmed by others. Using the staphylococcal clumping test (SCT) for FDPs and the serial dilution protamine sulphate test for fibrin monomer complexes, Gurewich *et al.*[69] showed that all patients with symptomatic, venographically

proven DVT had at least one test positive and 96% of their patients with proven PE had positive SCTs. Ruckley *et al.*[169] showed significantly elevated FDP concentrations in patients with PE but not in those with DVT alone and in 1972 Wood *et al.*[203] reported higher FDP concentrations in patients with congestive cardiac failure, sepsis or metastatic carcinoma than in those with DVT. Subsequently, Jones *et al.*[91] in a follow-up of Ruckley's work, confirmed the high levels in patients with PE, but there was a considerable overlap with patients who had other conditions. The highest concentrations were detected in those with disseminated intravascular coagulation. A possible explanation for some of the discrepancies found in the literature is provided by Hirsh *et al.*[79] In experimental pulmonary emboli in dogs they demonstrated that the high FDP concentrations were derived from lysis of fibrin deposited on the emboli rather than from the emboli, themselves. The elevated FDP concentrations could be prevented by treating the dogs with heparin. The concentration of FDP seems to be a measure of further fibrin deposition (or propagation) rather than of the established thrombus or embolus.

The measurement of FDP is, therefore, of some value in the diagnosis of PE but is insufficiently sensitive or specific to be useful in the diagnosis of DVT.

Platelet aggregation is another important aspect of thrombus formation. When they aggregate they release a variety of substances. This 'release reaction' can also be induced without aggregation and, in fact, platelets that have released may survive and perform normally in the circulation.[30,80,163] Release occurs

of both the contents of the granules and of substances located in the cytoplasm, mitochondria or membranes. Certain of the release factors, such as potassium, zinc, mucopolysaccharides, ADP, ATP and lysosomal enzymes are common to many different types of cell and are of little interest as indicators of thrombosis. Other substances, however, can be described as 'platelet specific' release products. I use specific as an operational definition, meaning that the activity or substance is antigenically distinct from any plasma protein and/or possesses a biological activity not found in native platelet poor plasma. Such platelet specific products could be indicators of intravascular thrombosis. As shown earlier, platelets are involved in the very earliest stage of thrombus formation and these release products could be extremely valuable as early indicators of thrombosis.

Various activities of these release products have been described, including antiplasmin and antiplasminogen activator,[127] heparin neutralising activity,[34] vascular permeability enhancing factor,[136] tissue culture stimulant factor[165,202] and thromboplastic activity.[186] The thromboplastic activity, platelet factor 3 (PF_3) was recognised as a property of the membrane lipoproteins.[90] Heparin neutralisation is the activity of one of the granule release proteins - platelet factor 4 (PF_4) and a radioimmunoassay has been developed.[19] Some doubt exists, however, as to whether the plasma heparin neutralising activity is assessing the same substance as the radioimmunoassay for PF_4. Using the heparin thrombin clotting time (HTCT) to measure plasma heparin neutralising activity (HNA), Ludlam *et al.*[110] showed that patients

with myocardial infarction had markedly elevated HNA but normal PF_4. They suggested that the HTCT measured HNA of other plasma proteins as well as that of PF_4. Niewiarowski[140] used a technique of isoelectric focusing to separate released platelet proteins and described a 'low activity' form of PF_4 which was immunologically distinct and suggested that it could be converted to β thromboglobulin by proteolysis.

Significantly increased concentrations of PF_4 were detected in the plasma of patients with DVT 7 - 240 days after onset of symptoms by O'Brien *et al.*[146] who used the HTCT. There was, however, a considerable overlap with the control group. Okuno and Crockatt[148] showed that only 30% of patients within 4 months of a DVT had elevated PF_4 as well as a similar proportion of patients with recent myocardial infarction. However, Ludlam *et al.*,[110] although confirming the raised PF_4 concentrations in patients with DVT, could not demonstrate elevated concentrations in those with MI.

A major criticism of the papers of both Okuno and O'Brien[146,148] is that the DVT patients were not all studied in the acute phase of their illness (i.e. as soon as symptoms occurred) and, therefore, they may have been studying patients who were not actually laying down thrombus. A further problem is that some of the patients were anticoagulated and we have shown that plasma concentrations of PF_4 measured by radioimmunoassay are markedly elevated following heparin injection.[40]

Therefore, with the doubt about what one is actually measuring with the heparin neutralising activity or the radioimmunoassay for PF_4 and the considerable overlap between patients with and without

thrombosis, the assessment of PF_4 is still unsatisfactory in the diagnosis of the thrombotic disease.

IDENTIFICATION OF BETA-THROMBOGLOBULIN AND DEVELOPMENT OF THE RADIOIMMUNOASSAY

Grette[67] reported the presence of proteins in the soluble material released during thrombin induced aggregation of washed human platelets and Davey and Luscher[37] showed that this material contained platelet specific alpha and beta globulins. In 1975 an attempt was made to characterise these proteins.[127] Moore *et al.* used platelet suspensions less than 24 hours old obtained from the SE Scotland Regional Blood Transfusion Service. These platelet suspensions were washed to remove plasma proteins, suspended and, after aggregation by bovine thrombin, the supernatant was removed for analysis. Preliminary chromatography on a Biogel A15M column separated the low and high molecular weight proteins. Further separation of the low molecular weight proteins on a Sephadex G200 column produced three distinct peaks. Peak 1 corresponded to IgG and peak 2 to albumin but peak 3 did not correspond to any known plasma or serum component. Electrophoresis on cellulose acetate confirmed that peak 3 consisted of a globulin with mobility between that of the beta and gamma globulins. Using sucrose density gradient ultracentrifugation, a sedimentation coefficient of 3.0 was calculated for the peak 3 proteins giving a molecular weight of 35,800. Immunodiffusion experiments using rabbit antiserum detected one major and at least three minor platelet antigens not

detected in fresh human plasma. The major antigen which became known as beta-thromboglobulin (βTG) was also detected in serum prepared from fresh whole blood, in the supernatant from 48 hours old platelet rich plasma and in plasma from citrated whole blood stored for 28 days at 4^0C. However, βTG could not be identified in the supernatant of thrombin aggregated, washed, 48 hours old platelets suggesting that it is released from the platelets during aging *in vitro*. With the immunodiffusion experiments two precipitation lines were seen in some samples indicating the possibility of two antigenically distinct forms of βTG.

Using the isolated βTG, Ludlam *et al.*[106] developed a radio-immunoassay which could detect plasma βTG in the range 2.5 - 150 ng/ml. They studied the release of βTG during platelet aggregation and showed that there was a small release associated with warming the sample from 0^0C to 37^0C, followed by a massive release as the platelets aggregated in response to collagen. Platelet poor plasma from normal individuals had concentrations of 19 ± 7.5 ng/ml with much higher concentrations in serum (17,400 ± 6,300 ng/ml) and the supernatant from platelet transfusion concentrates (247,000 ± 120,000 ng/ml). Studies of patients with a recent history of thromboembolism (venous and arterial) diagnosed clinically, showed elevated concentrations relative to the normal patients studied. Of 17 patients with prosthetic heart valves, 9 had levels greater than the normal range. They did, however, add the cautionary comment that βTG may not be a constituent of normal plasma but may be merely a manifestation of platelet release

occurring during sampling. Later that year Ludlam *et al.*,[107] using a rapid refined version of the standard immunoassay, investigated a group of patients with proven venous thromboembolism. They showed that 6 patients with evidence of DVT on phlebography or ^{125}I fibrinogen uptake tests all had elevated βTG concentrations but a further 8 patients without evidence of DVT had similar concentrations to a group of 35 normal individuals. They suggested that the βTG assay may be of value in the diagnosis of DVT.

The following year Bolton *et al.*[18] described further refinements of the βTG assay including a sensitive and a 'rapid' version of the standard assay, which had been described initially by Ludlam *et al.*[107] Further investigations into sampling and processing of blood specimens for βTG were reported by Ludlam and Cash.[108] They showed that the use of a mixture of EDTA, theophylline and prostaglandin E_1, with the blood cooled to 0-4^{0}C, resulted in the lowest βTG concentrations. If the blood was kept at this temperature, centrifugation could be delayed for up to 48 hours. Centrifugation of the blood at 1,900 *g* for 60 minutes seemed to be essential as it produced results significantly lower than centrifugation for 30 minutes but centrifugation beyond 60 minutes did not result in any further reduction in βTG concentration. If the blood was retained in the syringe, a significant release did not occur until 7 minutes had elapsed and, therefore, during a normal venepuncture, this was unlikely to contribute to the βTG concentration. They also showed that injecting blood at pressure through needles as fine as 23 guage did not result in significant release.

In 1978 Dawes *et al.*[40] showed that βTG was present in synovial and amniotic fluid in slightly higher concentrations than in plasma. As platelets are not present in either of these fluids, it is suggested that plasma βTG concentrations are not, in fact, due purely to release during sampling but are a normal constituent of body fluids.

Further studies on the chemical structure of βTG by Begg *et al.*[11] have shown that βTG consists of four identical subunits of amino acids with molecular weights of 8,851. This correlates well with the molecular weight of 35,800 for the whole molecule determined by Moore *et al.*[127] by ultracentrifugation. Begg *et al.*[11] also found that the structure of PF_4 was remarkably similar to βTG in that 42 of the 81 residues of βTG are identical to PF_4. This may support the contention of Niewiarowski[140] that 'low activity' PF_4 is converted to βTG by proteolysis.

These researches suggest that βTG fulfils the criteria of a 'platelet specific' release factor. It is normally present in the body fluids in small amounts and is released during the process of platelet aggregation. Provided that blood samples are taken with care and rapidly cooled and stabilised in the anticoagulant mixture, the measured concentration of βTG will accurately represent the *in vivo* plasma concentration.

As platelet aggregation is a very early event in the development of a thrombus, the βTG assay, unlike many of the other tests for thromboembolism, has the very exciting potential of diagnosing intravascular thrombosis at its inception.

AIMS OF THE STUDY

In view of the interesting preliminary clinical data produced by Ludlam[107] it was evident that there was a need to study further the use of the βTG assay in clinical situations. First, however, it was necessary to carry out some further investigations into the sampling techniques, the clearance of βTG from the circulation and to establish normal ranges for plasma and urine βTG.

The clinical study is divided into three parts. The first part is concerned with the use of the plasma and urine βTG concentrations to diagnose venous thromboembolism and to assess their relative diagnostic accuracy. In the second part the value of the βTG assay in the diagnosis of peri-operative thrombotic complications is assessed. Finally, the βTG concentrations in patients with atherosclerosis were studied.

CHAPTER I

STUDIES ON THE SAMPLING OF BLOOD FOR PLASMA BETA-THROMBOGLOBULIN AND OF ITS PLASMA AND URINARY KINETICS

1. SAMPLING METHODS

Platelet release occurring during sampling is a potential limitation to the clinical value of the assay. The aim of this phase of the project was to examine potential sources of error associated with the withdrawal and storage of blood.

Ludlam[106] studied the effects of various substances in the prevention of βTG release during sampling and concluded that a mixture of EDTA, theophylline and prostaglandin E_1 gave the most reliable results. However, as prostaglandin E_1 is expensive and must be stored in a deep freeze, it was wished to eliminate it, if possible, from the collecting medium.

The physical process of withdrawing blood from the subject may stimulate platelet release. A possible solution was to have the anticoagulant mixture in the syringe so that the blood is immediately stabilised. Other potential influences are indwelling catheters which are commonly used for blood sampling, and venous stasis which is known to activate the fibrinolytic system.

Experiment i: The platelet stabilising anticoagulant mixture

Methods:

Samples of blood were obtained from eight normal subjects and three patients with proven venous thromboembolic disease. Through

a 19 gauge needle 30 ml of blood were drawn into a polypropylene syringe and aliquots were inserted into siliconised glass tubes containing three different anticoagulant and platelet stabilising mixtures. The first set of tubes contained 0.1 ml of disodium ethylene diamine tetra-acetate (E) in a concentration of 100 mg/ml; the second, E and 0.1 ml theophylline in a concentration of 5.4 mg/ml (ET), and the third contained ET plus 0.1 ml of prostaglandin E_1 in a concentration of 1 µg/ml (ETP). The tubes were cooled in melting ice before venesection and were filled immediately after venepuncture in a different order for each subject. Four tubes of each of the three mixtures were filled from every venepuncture. The tubes were transported in melting ice and were maintained at 4^0C until plasma separation. One tube of each anticoagulant mixture from each subject was centrifuged at 4^0C within 1 hour of sampling and the top third of the plasma was withdrawn and deep frozen prior to assay in batches. The other three tubes from each group were similarly processed at 24 hour intervals up to 72 hours.

Results:

There was little difference in the normal individuals between blood samples stabilised in ET and ETP up to 24 hours after sampling (Fig. 2). Those stabilised in E alone were, however, much higher by 24 hours. In the three DVT patients ETP performed much better than E or ET which both failed to stabilise the platelets up to 24 hours.

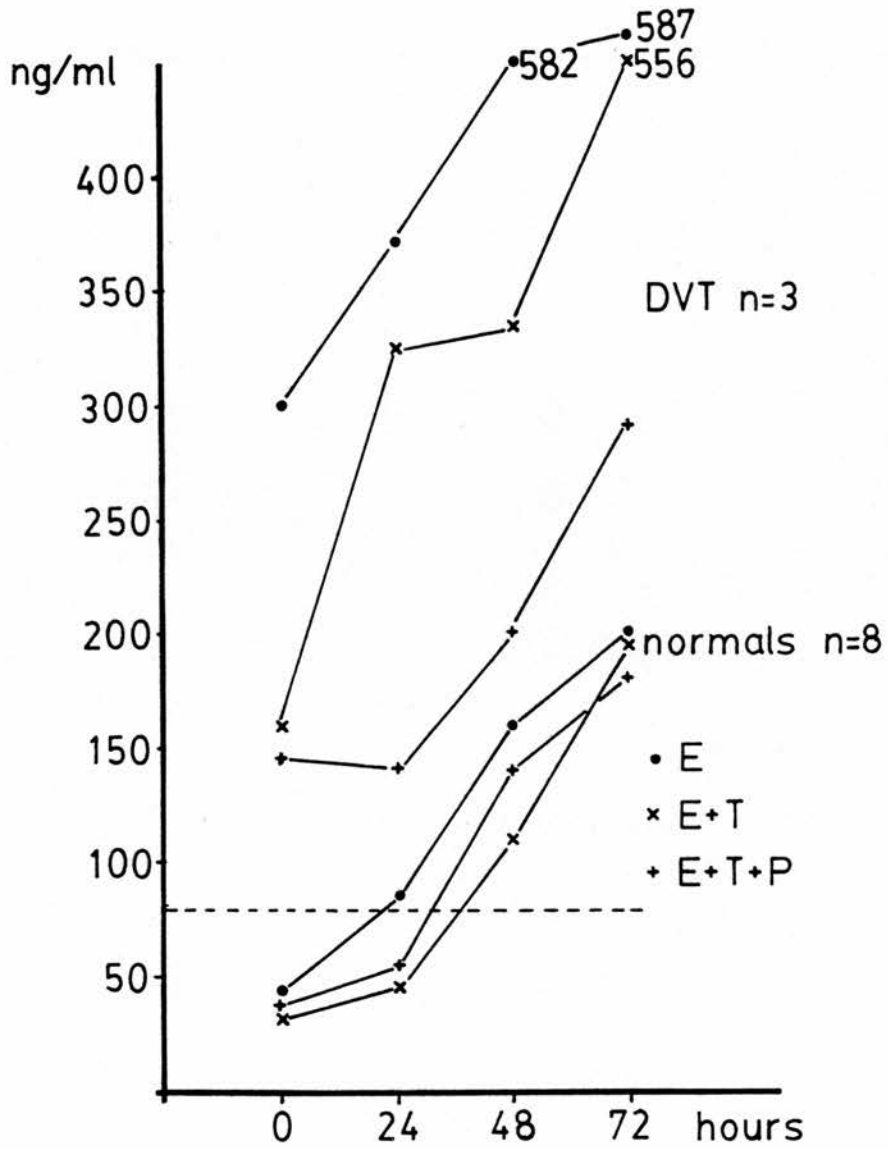

Fig. 2: The stabilising effect of three different anticoagulant mixtures in 8 healthy subjects and in 3 patients with DVT. Median plasma βTG concentration plotted against time to plasma separation.

Experiment ii: The anticoagulant mixture in the syringe

Methods:

Two samples of blood were obtained simultaneously from each of 20 healthy volunteers. A sample from one arm was withdrawn in the normal fashion and placed in a cooled tube containing the anti-coagulant mixture. A second sample was drawn from the other arm into a syringe containing the cooled anticoagulant mixture and then placed in a cooled tube. Both sets of samples were then processed in the normal way.

Results:

The effect of loading the syringe with the stabilising mixture is shown in Fig. 3. The difference in the concentrations was not significant ($p > 0.1$, Wilcoxon). The median of the group with ETP in the tube was 24 ng/ml and of the group with ETP in the syringe was 21 ng/ml. One spuriously elevated value of 260 ng/ml was obtained from the group with ETP in the tube. The ETP in the syringe appeared to eliminate these spuriously high results.

Experiment iii: The effect of venous stasis

Methods:

Blood samples were drawn in the normal way without venous stasis from 11 healthy volunteers. A venous tourniquet, inflated to 60 mm Hg was then placed on the other arm and a second blood sample was drawn from this arm after 3 mins of venous stasis.

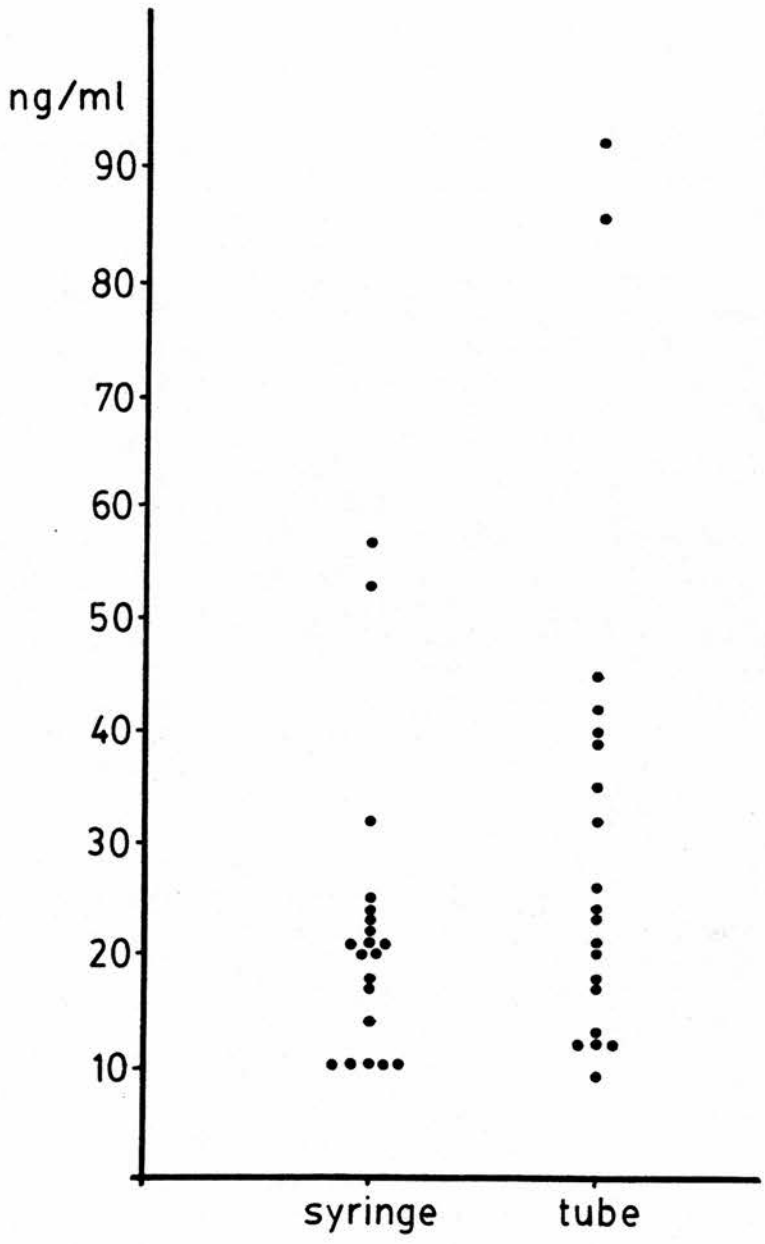

Fig. 3: The effect of putting the platelet stabilising mixture into the syringe.

Results:

The effects of venous stasis are shown in Fig. 4. There was no significant difference in βTG concentration after 3 mins of venous stasis ($p > 0.1$, Wilcoxon).

Experiment iv: Indwelling venous catheters

Methods:

Two normal volunteers and 3 patients with arterial disease were studied. The latter group underwent central venous catheterisation to facilitate monitoring during arterial reconstruction. A blood sample was drawn using the standard technique from one arm and a central venous catheter (Abbott Drum-cath) was then inserted, using local anaesthesia (2% Lignocaine) into an ante cubital vein in the other arm. The central venous catheter was continuously perfused with 0.9% saline through a 3 way tap to prevent reflux of blood. Blood samples were withdrawn every half hour for 3 hours, from the catheter and by individual venepuncture from the other arm. Each successive venepuncture was performed more distally on the arm to avoid the effects of local platelet activity at the venepuncture sites. The blood sample from the catheter was withdrawn through the 3 way tap after first withdrawing and discarding 5 ml of blood to ensure that the catheter system was full of fresh venous blood. The catheter was flushed with saline after sampling to prevent clots accumulating in the system.

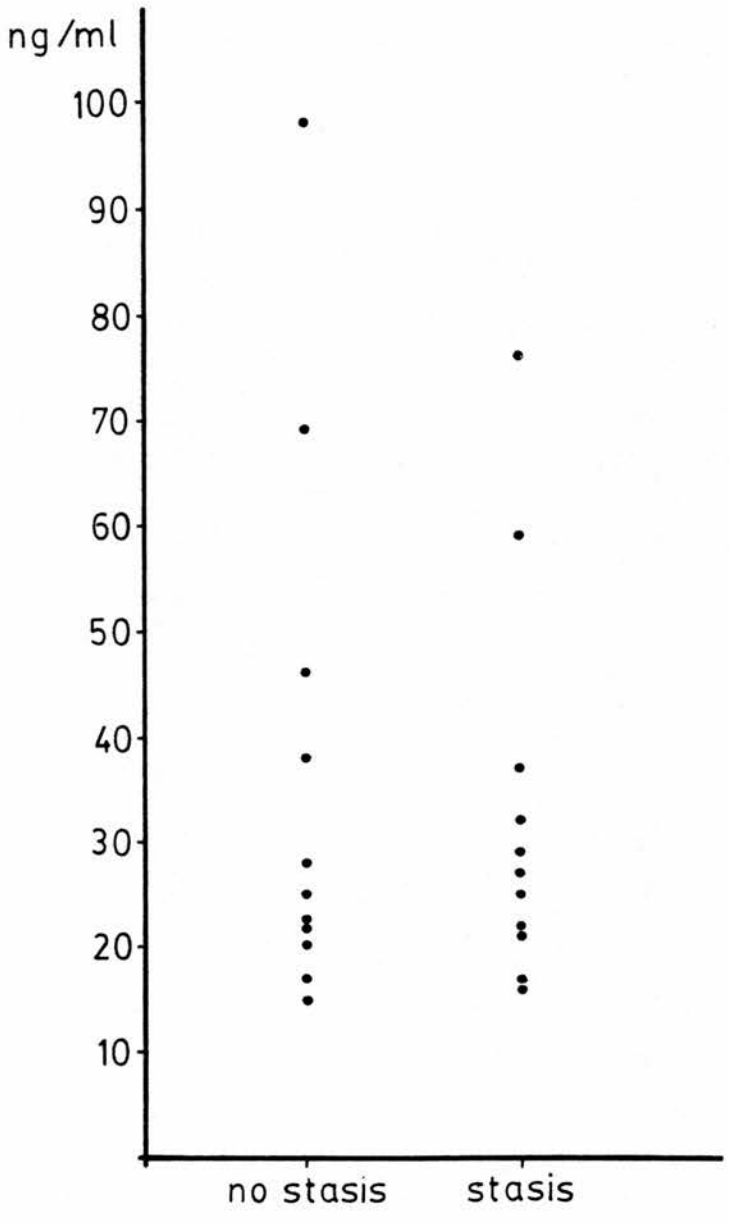

Fig. 4: Venous stasis and plasma βTG concentration.

Results:

Table 1 compares venepuncture with catheter sampling. In the samples taken by individual venepuncture only one sample (3%) exceeded 80 ng/ml whereas in the samples drawn through the catheter, 9 (26%) had concentrations exceeding 80 ng/ml. This difference was significant ($\chi^2 = 5.72$, $p = 0.017$). Using a paired t test the concentrations obtained by venepuncture were significantly lower than those obtained through the catheter ($t = 3.61$, $p = 0.001$). The values in the individual venepuncture series were not different from our normal range and there was no tendency for the βTG concentrations to rise as the experiment progressed.

Discussion

Our findings in experiment i confirm that it is of value to use EDTA, theophylline and prostaglandin E_1 as the platelet stabilising anticoagulant cocktail. Although prostaglandin E_1 contributed little when sampling from normal individuals it did prolong the period before release occurred with storage in patients with DVT. This may be due to a need for an enhanced antiaggregating effect in patients with DVT who have been shown to have an increased platelet adhesiveness.[77,178] Considerable release occurred, regardless of anticoagulant used in all samples after 24 hours and, therefore, samples should be processed within 24 hours of sampling.

The addition of the anticoagulant mixture to the syringe did not significantly affect the results obtained. The mean concentration when the mixture was in the tube was distorted by one rogue

TABLE 1: SAMPLING BLOOD FOR PLASMA βTG CONCENTRATION THROUGH INDWELLING CATHETERS AND BY INDIVIDUAL VENEPUNCTURES (CONCENTRATIONS IN ng/ml)

Subject	Individual Venepunctures			Catheter Samples		
	No. >80 ng/ml	Range	Mean	No. >80 ng/ml	Range	Mean
Normal A	0	16-34	24.0	2	26-221	70.9
Normal B	0	16-37	26.7	1	22-104	41.4
Patient C	0	18-47	29.8	4	51-262	122.1
Patient D	0	15-66	29.8	0	18-48	30.9
Patient E	1	28-83	49.7	2	38-110	67.4

concentration (260 ng/ml). This is likely to be due to thrombin generation inducing release in the syringe and in that particular individual was prevented in the second specimen which had the anticoagulant mixture in the syringe. However, although the presence of the mixture in the syringe appeared to eliminate these spuriously high results, the difference in concentration was not sufficient to justify adopting this more expensive and inconvenient method of sampling.

Venous stasis, similarly, did not affect the results obtained. Sampling through long catheters proved to be unreliable although the presence of the catheter does not appear to raise systemic βTG concentrations as demonstrated by separate venepunctures. This suggests that βTG release occurs actually in the catheter and is probably due to the formation of small thrombi in the junctions between the catheter and the 3 way tap despite flushing with saline. Blood samples for βTG assay should, therefore, be taken by individual 'clean' venepuncture and not through an indwelling catheter. As the presence of the catheter did not affect the systemic concentrations of βTG, it is unlikely that the small catheters used to give intravenous fluids will significantly affect the βTG concentrations in clinical studies.

2. NORMAL RANGES

Prior to investigating βTG concentrations in pathological situations it was essential to ascertain the normal ranges of plasma and urine in samples assayed in our local radio-immunoassay laboratory. The assays were performed by the technique developed in Edinburgh by Ludlam *et al.*[106] and not by the commercially available assay kit which was developed out of the Edinburgh work. Several normal ranges quoted in the literature are summarised in Table 2.

The urinary assay became available only in the latter part of the study and, therefore, plasma and urine samples were obtained on a smaller group of healthy subjects.

Experiment i: Assessment of normal range of plasma βTG

Methods:

From 26 individuals who had no overt evidence of any pathological process, 94 plasma samples were obtained by separate 'clean' venepunctures. The ages of these individuals ranged from 20 to 59 years (mean 29.4, SD 11.3 years).

Results:

Figure 5 shows the normal plasma βTG concentrations. The range was 8.9 to 98.3 ng/ml with a mean of 29.3 ng/ml and standard deviation of 18.3 ng/ml. The median concentration was 23 ng/ml. The distribution was considerably skewed by the preponderance of results in the lower concentrations.

TABLE 2: NORMAL RANGES OF PLASMA βTG CONCENTRATIONS FROM THE LITERATURE (CONCENTRATIONS IN ng/ml)

Ref.	Date	Assay*	No. of samples	No. of subjects	Range	Mean	SD	Upper limit
106	1975	Ed.	13	-	-	19	7.5	-
107	1975	Ed.	35	35	30-58	38.7	9.1	-
42	1977	Kit	49	7	-	29.8	-	82
27	1977	Ed.	-	35	12-90	27.8	12.4	-
31 (Male)	1979	Kit	-	47	3-65	28.9	-	55.6
31 (Female)	1979	Kit	-	56	5-74	26.1	-	55.6
159	1977	Kit	53	7	-	27.6	-	-
159	1977	Kit	10	10	-	25.3	-	-
159	1977	Kit	32	6	-	24.8	-	-
159	1977	Kit	26	8	-	25.1	-	-
Present series		Ed.	94	26	9-98	29.3	18.3	80

* Assay: Ed. = assay in MRC radioimmunoassay laboratory, Edinburgh.
Kit = assay by the Radiochemical Centre RIA Kit.

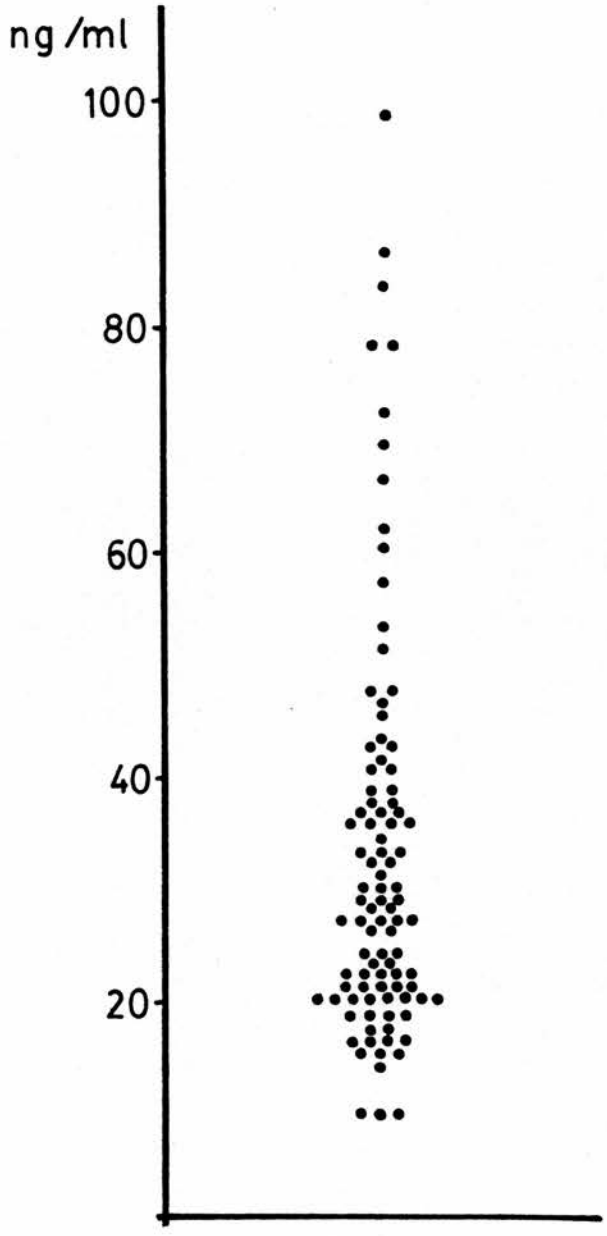

Fig. 5: Normal range of plasma βTG concentrations in 26 healthy subjects.

Experiment ii: The normal range of urinary βTG concentration and its relationship to plasma βTG concentration

Methods:

Twelve healthy volunteers (4 female and 8 male) aged 22-57 years (mean 30.8, SD 10.1 years) were studied for periods up to 60 hours. The total volume of each urine specimen passed and the period over which it was collected were recorded and an aliquot of 10 ml was taken for βTG assay. The specimens were then deep frozen and stored for assay in batches. This enabled calculations of the βTG concentration, the hourly urine output and the hourly βTG excretion to be made. At least one sample from each subject was tested chemically for blood and protein using a dipstick (Labstix, Ames), and all were negative. During the study periods 2 - 6 plasma samples were also obtained from each subject. A total of 81 urine specimens and 38 plasma specimens were obtained.

Results:

The distribution of urinary concentrations is shown in Fig. 6 and summarised in Table 3. The urine βTG concentration varied from <0.05 ng/ml to 0.46 ng/ml. In 54 of the 81 specimens the concentration was below the lowest detection limit of the assay and, therefore, it is impossible to calculate accurate values for the mean and standard deviation. There is no difference in concentration between males and females in both groups; the median concentration was below the assay limit.

The low concentrations also prevented an accurate measurement of the βTG excretion rate. In the 27 patients in whom the

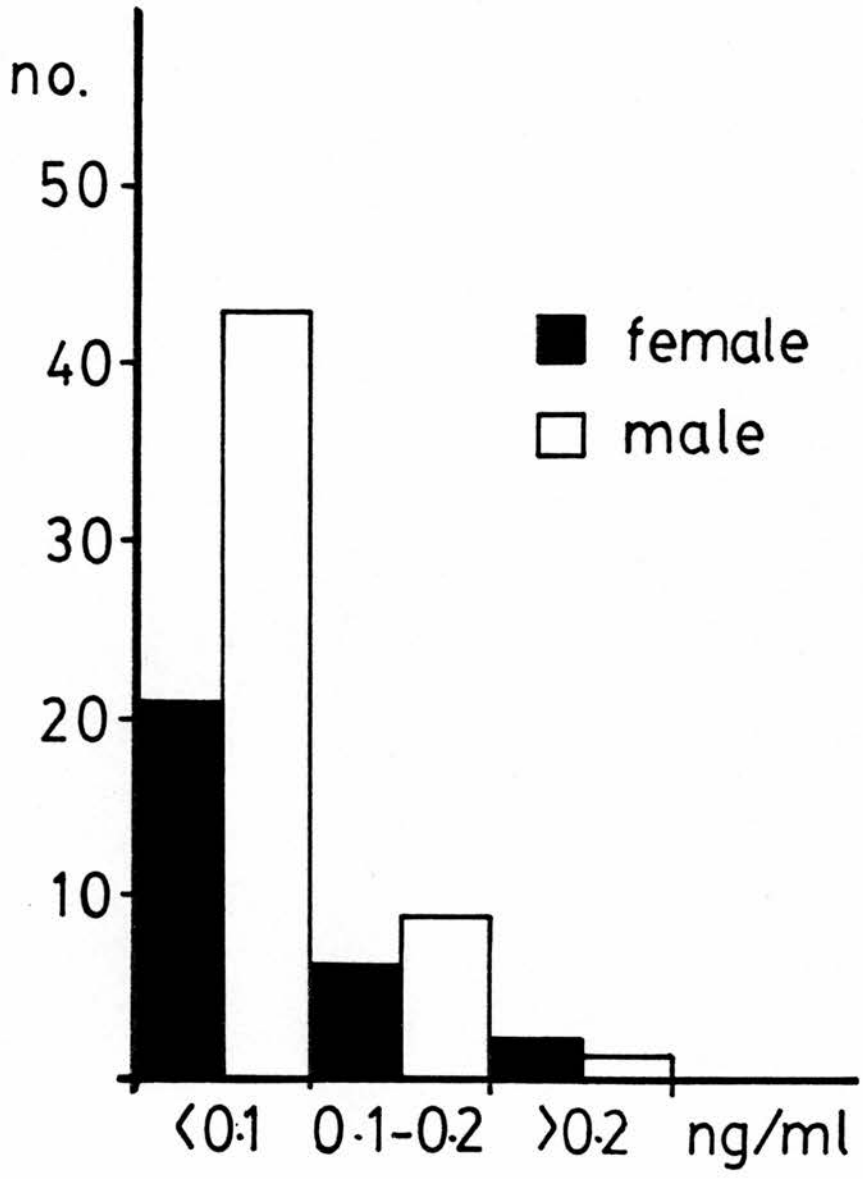

Fig. 6: Normal range of urinary βTG concentrations in 12 healthy subjects. Vertical axis represents the number of specimens.

TABLE 3: NORMAL RANGES OF URINARY βTG CONCENTRATIONS AND EXCRETION RATES

Sex	No. of subjects	No. of samples	Concentration range (ng/ml)	Excretion rate range (ng/hr)
Male	8	53	< 0.05-0.3	< 1.2-10.9
Female	4	28	< 0.05-0.46	< 2.7-13.8
Total	12	81	< 0.05-0.46	< 1.2-13.8

TABLE 4: RELATIONSHIP BETWEEN URINARY βTG CONCENTRATION AND URINE FLOW RATE. FIGURES REPRESENT NUMBERS OF SPECIMENS.

BTG concentration (ng/ml)	Urine flow rate (ml/hr)			
	< 50	50-100	> 100	Total
< 0.05	14	22	18	54
0.05-0.1	4	5	0	9
0.1-0.2	7	8	0	15
> 0.2	3	0	0	3
Total	28	35	18	81

concentration could be measured the excretion rate ranged from 1.2 to 13.8 ng/hr.

The hourly urine output varied from 18.5 to 1040 ml/hr. The relationship between the urine flow rate and the βTG concentration is shown in Table 4. In none of the specimens in which a measurable concentration was obtained did the urine flow rate exceed 100 ml/hr. However, concentrations below the assay limit were obtained in every specimen from 5 subjects even with flow rates below 50 ml/hr.

In the group of 27 specimens in which accurate βTG concentrations and excretion rates could be measured it is possible to calculate a correlation coefficient (r) for the different parameters measured. The r values are as follows:-

Urine flow rate (ml/hr) and urine βTG concentration (ng/ml)	0.40
Urine flow rate (ml/hr) and βTG excretion rate (ng/hr)	0.28
βTG concentration (ng/ml) and βTG excretion rate (ng/hr)	0.81

The βTG concentration in 38 plasma samples withdrawn during the period of sampling varied from 10.0 to 98.3 ng/ml (median 25 ng/ml). In one subject, who had two elevated plasma concentrations, the urine concentrations at the time of plasma sampling were below the assay limit, despite low urine flow rates (< 50 ml/hr) at the time. The subject, who had a considerably elevated urine concentration in one sample, had a normal plasma concentration (37.5 ng/ml) during that collection. There was no correlation between plasma and urine βTG concentrations ($r = 0.042$) or between the plasma concentration and the βTG excretion rate ($r = 0.043$).

The urine and plasma concentrations, urine flow rates and βTG excretion rates in one subject over a 60 hour period are shown in Fig. 7.

Discussion

The mean plasma βTG concentration in these subjects correlates well with those quoted in Table 2. However, in view of the skewed nature of the distribution of plasma βTG concentrations, the normal method of assessing the normal range (mean ± 2 x SD) gives a low upper limit. Therefore, 80 ng/ml has been selected as the upper limit of the normal range as this includes over 95% of the samples. This is in keeping with the 95% confidence limit of 82.2 ng/ml quoted by Denham *et al.*[42] Three samples in two subjects fell outside this limit. One subject was taking an oestrogen-containing contraceptive pill but had no overt evidence of thromboembolic disease. Nevertheless, elevated concentrations in patients on oestrogen-containing contraceptive pills but without apparent thrombotic disease have been reported.[50] The other two samples were from a male subject, apparently healthy, who showed elevated concentrations on several subsequent occasions but with no apparent cause. 'Syringe' testing (reported later) was carried out on this subject and he showed no tendency to more rapid release than the other subjects studied. βTG could be detected in all the plasma samples.

There is some doubt as to whether the βTG detected in the urine is the complete molecule or merely peptide fragments

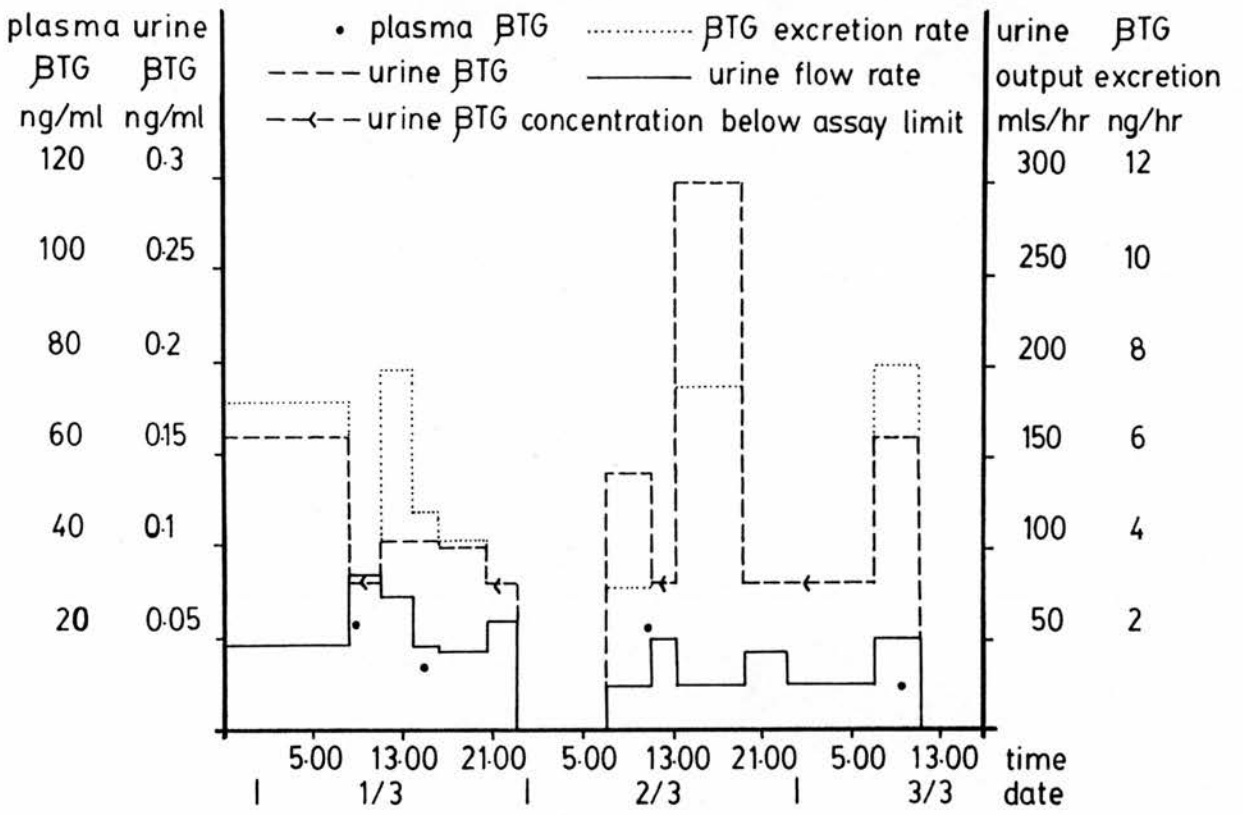

Fig. 7: Urine and plasma βTG concentrations, urine flow rates and βTG excretion rates in a healthy subject over 60 hours.

containing the antigen site. However, if one can correlate what is measured in the urine with the plasma βTG, it is immaterial whether this is the entire molecule or merely a fragment.

Urine sampling is potentially more attractive than plasma sampling because it is not prone to artefacts of sampling and processing. However, it is likely to be affected by haematuria and possibly also proteinuria. At least one sample from each subject was tested for blood and protein and all were negative.

As with plasma samples, the urine samples showed a skewed distribution with two-thirds of the samples having concentrations below the assay limit. An upper concentration limit was, therefore, set to include at least 95% of the specimens. This gave an upper limit of the normal range of 0.21 ng/ml.

This correlates well with the upper limit of the normal range (0.25 ng/ml) quoted by Anderton *et al.*[4] The measured excretion rate varied from 1.2 to 13.8 ng/hr in the 27 specimens in which it could be accurately calculated. However, an upper limit of 15 ng/hr will include all but two of the specimens, even if the excretion rate in the unmeasurable specimens is calculated on the lowest detectable concentration on the assay. This we propose as the upper limit of the normal excretion rate.

All the urine specimens obtained during periods of urine flow > 100 ml/hr were associated with βTG concentrations below the assay limit but with flow rates of < 50 ml/hr only half of the specimens had unmeasurable concentrations. All subjects, however, could dilute the βTG concentrations below the assay limit with minimal

diuresis.

In the 27 samples with measurable concentrations the βTG concentration correlated more closely with the βTG excretion rate ($r = 0.81$) than with the urine flow rate ($r = 0.40$). This suggests that, provided the urine flow rate is not in excess of 100 ml/hr, the urine concentration of βTG is a reasonable indicator of the βTG excretion rate. At higher urine flows in normal subjects the dilution effect negates the value of the βTG concentration. We were unable to demonstrate any correlation between the plasma concentration and either the urine concentration or the excretion rate.

In one subject with high plasma concentrations the urine concentration never reached measurable levels despite urine flow rates as low as 33 ml/hr. Another subject, however, showed a urine concentration of 0.46 ng/ml at a similar flow rate (31 ml/hr) and a plasma concentration less than half that of the previous subject. However, in normal subjects one is dealing with relatively narrow ranges of βTG concentrations and excretion rates. In this situation demonstration of correlation becomes more difficult. Another important factor is that the plasma βTG measures the βTG concentration at a moment in time whereas the urine βTG reflects its excretion over the period that the urine was accumulating in the bladder, which may be several hours. If one can demonstrate that increased release of βTG in the body relates to increased excretion in the urine, the urinary assay is potentially more valuable as it will be affected less by any transient changes in plasma βTG. It could reflect more accurately the platelet activity over a period of time than a single plasma assay.

3. RELATIONSHIP OF PLASMA BETA-THROMBOGLOBULIN CONCENTRATION TO PLATELET COUNT AND SERUM BETA-THROMBOGLOBULIN CONCENTRATION

Since βTG is released from platelets, the platelet count may influence the plasma βTG concentration. If this is so, the interpretation of any plasma βTG concentrations would need to take account of variations in the platelet count. Similarly, the actual βTG content of the platelets may also affect the plasma βTG concentration. If blood is allowed to clot, maximal release from the platelet occurs and, therefore, the assessment of the βTG concentration in serum will reflect the βTG content of the platelets.

Experiment i: The relationship between plasma βTG and platelet count

Methods:

a) Platelet counts are available on 72 out of the 94 specimens from healthy individuals.

b) In a patient with alcoholic thrombocytopenia, daily βTG concentrations and platelet counts were obtained during her hospital stay.

Results:

a) The βTG concentrations plotted against the platelet counts are shown in Fig. 8. This gives a correlation coefficient of $r = 0.46$. There is, therefore, a slight positive correlation between the platelet count and the plasma βTG concentration in healthy subjects. The relationship is defined by the

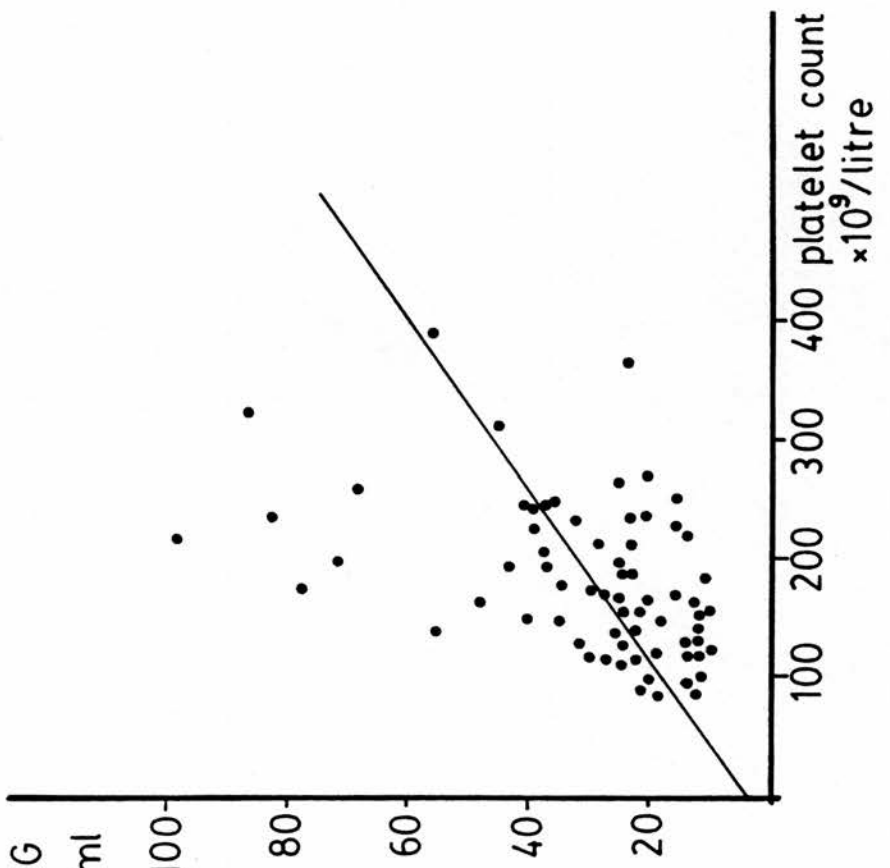

Fig. 8: Plasma βTG concentration v platelet count.

following equation:

Plasma βTG (ng/ml) = 3.6 + 0.14 x Platelet count x 10^9/litre

The regression line is shown in Fig.8.

b) Fig. 9 shows the parallel rise in platelet count and the βTG concentration during the recovery from alcohol induced thrombocytopenia. This correlation continued for the first 7 days but the βTG concentration then fell despite maintenance of the platelet count.

Experiment ii: The relationship between βTG concentration in plasma and serum

Methods:

Blood samples were obtained from 13 healthy volunteers and aliquots were processed for plasma βTG assay. Further specimens from each subject were allowed to clot and, after suitable dilution of the serum, βTG assays were performed.

Results:

The serum βTG concentrations were approximately 1,000 x the plasma concentrations (Table 5). There was no consistent relationship between the plasma and the serum βTG concentrations ($r = 0.15$), nor between the platelet count and the serum βTG concentration ($r = 0.10$), nor between the plasma βTG concentration and the serum concentration corrected for the platelet count ($r = 0.16$).

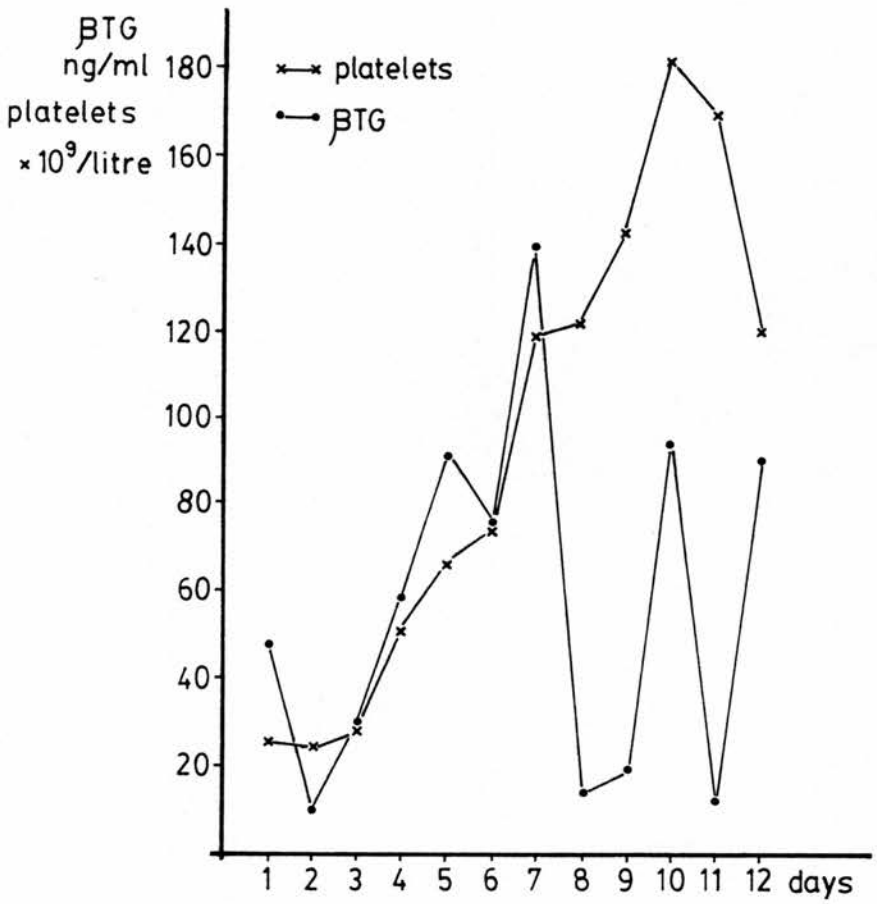

Fig. 9: Plasma βTG concentration and platelet count during recovery in a patient with alcohol induced thrombocytopenia.

TABLE 5: PLASMA βTG CONCENTRATIONS, SERUM βTG CONCENTRATIONS AND PLATELET COUNTS

	Range	Mean	SD
Plasma βTG ng/ml	8.9-32.0	15.5	7.8
Serum βTG µg/ml	10.2-22.4	17.4	3.6
Platelet count x 10^9/litre	100-182	134.2	25.1

Discussion

There appeared to be a poor correlation between the platelet count and the plasma βTG concentration (r = 0.46). Fig. 8 suggests that the correlation is better at low platelet counts than at high platelet counts and this is borne out by the findings in the patient with alcohol induced thrombocytopenia (Fig. 9). Turpie[184] also reported a case of marrow aplasia with no detectable circulating platelets in whom βTG could not be detected in the plasma. However, in other patients with thrombocytopenia, due to destruction of platelets the plasma βTG may be normal in the autoimmune type or even elevated in the thrombotic type.[73]

The correlation between the serum and the plasma βTG concentrations was very poor even after correction for the platelet count and so it seems that the plasma βTG in healthy subjects varies in response to factors other than the platelet count and the platelet content of βTG.

Another interesting feature in the patient with alcoholic thrombocytopenia was that the βTG concentration rose in parallel with the platelet count even into the abnormal range (140 ng/ml). One could have expected that the plasma βTG concentration would cease to rise when the normal range was reached. A possible explanation of this is that the majority of the platelets will be young, large platelets which have been shown by Karpatkin[98] to be very much more active and to release more PF_4 than small, old platelets. As the platelets age they become less active and the fall in plasma βTG on day 8 may be a manifestation of an increasing

population of older platelets with a reduction in the rate of production of new platelets. With a high proportion of young platelets, the measured βTG concentration may be a less accurate assessment of the plasma βTG concentration as these active platelets will be more likely to release during sampling.

This study indicated that, provided the platelet count approximated to the normal range, there was no need to correct the plasma βTG for the varying platelet count.

4. SYRINGE TESTS

Clumsy technique and delay during sampling can result in spuriously high plasma βTG concentrations due to release occurring during the process of sampling.[108] It was necessary, therefore, to determine how long blood could be kept in a syringe before significant release occurred and whether there was any difference between individuals. Nossel[141] has shown that it takes approximately 7 mins before blood in a syringe will show a significant rise in fibrinopeptide A concentrations. This suggests that there is a delay before sufficient thrombin is generated to convert fibrinogen to fibrin. This test, therefore, has potential value in assaying the rate of thrombin generation and, if one uses the βTG assay, it may also assess the sensitivity of the platelets to thrombin.

Experiment i: Identification of normal release patterns

Methods:

Ten healthy volunteers aged 19-41 years (mean 28.5 years, SD 7.1 years) were studied. Through an 18 gauge needle 30 ml of blood were drawn into a polypropylene syringe and 2.7 ml aliquots were inserted into cooled tubes containing the anticoagulant cocktail, at 1 min intervals. In all cases the venesection took < 1 min. The samples were then processed in the normal fashion.

Results:

The results are shown graphically in Fig. 10. There appear to be two different patterns of release - an early group (A,B,C,J & K)

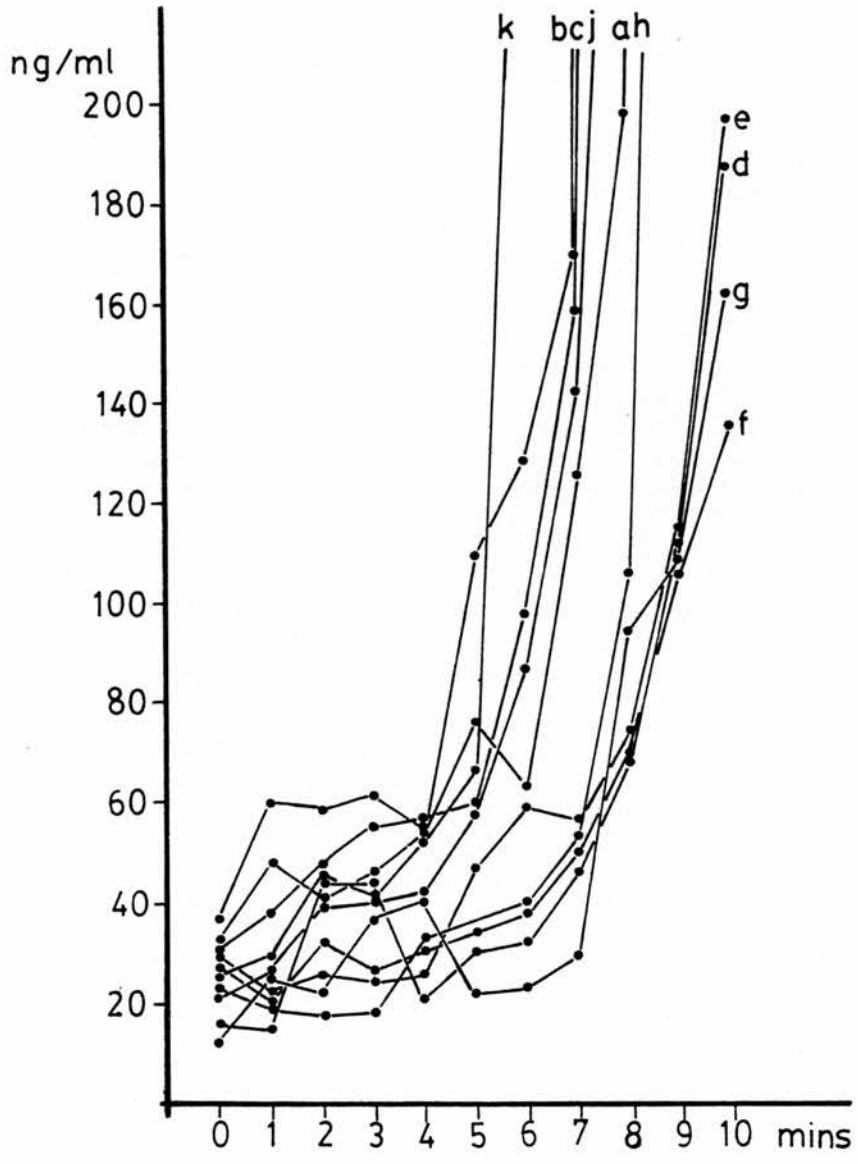

Fig. 10: Syringe tests on 10 healthy subjects.

and a late group (D,E,F,G & H). In order to compare syringe tests on different individuals a point was selected, designated the 'Release Time', at which the amount of βTG released was likely to be significant. This was the time at which the plasma βTG concentration had risen by at least 50 ng/ml above the baseline. The baseline was defined as the lowest plasma βTG concentration detected and was not always in the first specimen. The slow release group (type I) had release times of > 7 mins (mean 8.2 mins) and the rapid release group (type II) had release times of 5-6 mins (mean 5.6 mins). The release times of the two groups are summarised in Fig. 11.

Experiment ii: Syringe test reproducibility

Methods:

In 3 volunteers the syringe test was repeated on two occasions.

Results:

The reproducibility was poor (Table 6). The release time tended to be shorter with higher basal concentrations.

Discussion

The pattern of release in normal subjects shows an almost horizontal line initially indicating that the release reaction has taken some time to become established. After a period varying from 4 - 9 mins it suddenly progresses rapidly. These results correlate well with those of Prowse *et al.*[157] who selected the point at which the baseline concentration plus 100 ng/ml was reached

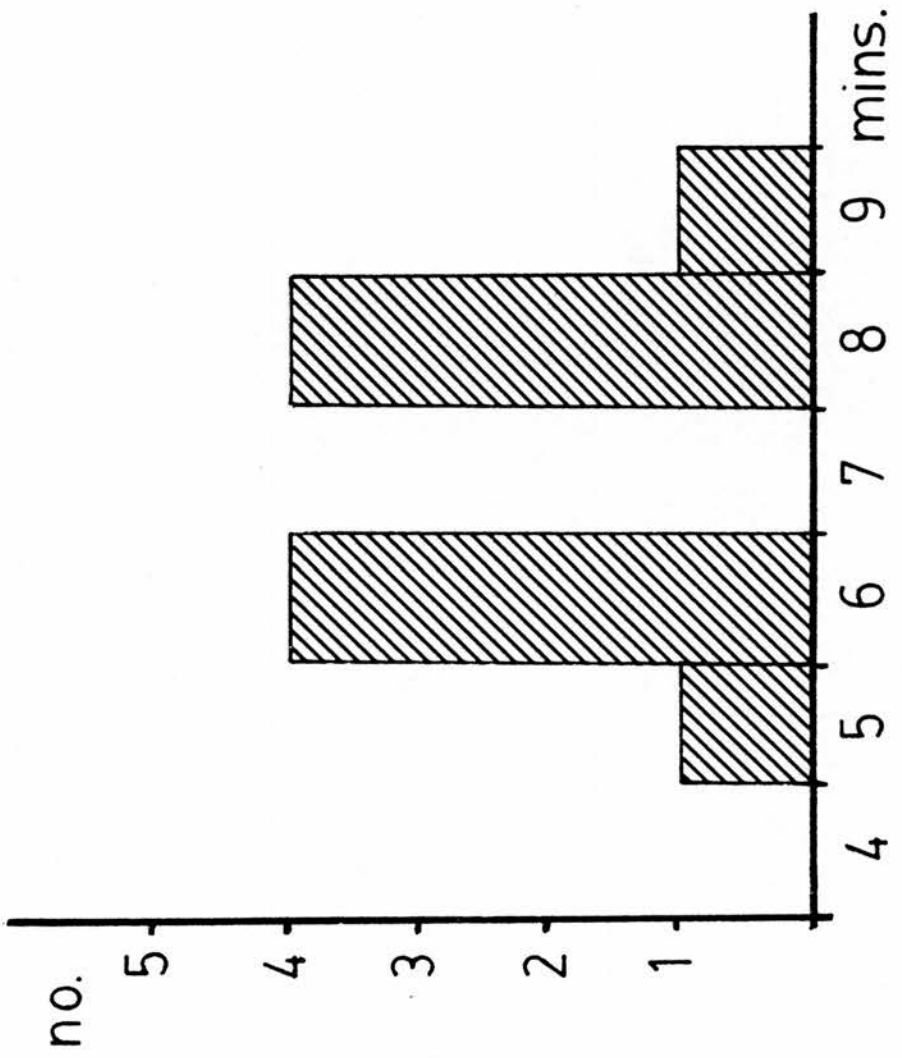

Fig. 11: Release times for the syringe tests in 10 healthy subjects.

TABLE 6: SYRINGE TEST REPRODUCIBILITY

Subject	A		B		C	
Test	Release time (mins)	Basal concentration (ng/ml)	Release time (mins)	Basal concentration (ng/ml)	Release time (mins)	Basal concentration (ng/ml)
1	6	27	3	29	9	32
2	7	26	6	26	5	58
3	5	26	6	36	1	136

as their release time. They also showed a bimodal distribution with rapid and slow types of release. Their patients showed a very close correlation between βTG and FPA release lending support to the theory that platelet release in this situation is due to thrombin generation. Nossel[143] has shown a similar parallel release of βTG and FPA, which he attributes to thrombin generation, after intrauterine infusions of hypertonic saline. Prowse *et al.*[157] also showed that FPA generation in the syringe test could be prevented by having a high concentration of heparin in the syringe. This, however, did not prevent βTG release but merely delayed it. They suggest that the high affinity of thrombin for platelets[65] relative to fibrinogen[187] and the resistance of platelet bound thrombin to plasma inhibitors[113] may permit thrombin induced release even in the presence of heparin. The reason for the two release patterns is obscure. It could be due to a differential binding of thrombin to the two platelet populations. The tighter binding might render the platelets more ready to release. Alternatively, there might be a different ease of thrombin production by the two populations. However, there is no evidence in studies of thrombosis to suggest two discrete rates of thrombin generation. Finally, there may be two different populations of platelets with different sensitivities to thrombin. This could be of significance in the clinical studies where spurious release was occasionally noticed. Platelets of the rapid release type will probably be more sensitive to errors of processing and sampling.

The reproducibility of the tests in the three subjects in experiment ii was rather poor, in contrast to the rather better

reproducibility obtained by Prowse *et al.*[157] The tests which had high baseline concentrations (samples 2 and 3 in subject 3) also tended to have more rapid release, 5 and 1 mins. This may be due to the presence of more active platelets at that time in the subject. In subject 1, however, there was a variation in the release time despite a constant baseline concentration.

The results of these syringe tests suggest that during normal sampling there is unlikely to be significant release occurring in the syringe.

5. EFFECT OF ASPIRIN AND HEPARIN ON PLASMA AND URINE BETA-THROMBOGLOBULIN CONCENTRATIONS

Aspirin is known to inhibit the platelet release reaction *in vitro* by the acetylation of cyclo-oxygenase which prevents the formation of the cyclic endoperoxides from arachidonic acid.[166] A single dose of Aspirin can produce a platelet defect lasting 7 to 10 days[111] and Quick[158] has shown that it will prolong the bleeding time. The dose of Aspirin also appears to be critical as the maximum effect may be obtained with a dose as low as 325 mg.[25] Heparin is also known to affect platelet function although there is much contradictory and inconclusive evidence as to its actual effect.[135] Both these drugs were used in our patients: Aspirin in those with carotid artery disease and Heparin in patients with venous thromboembolism and in those undergoing vascular surgery. It was, therefore, of interest to assess the effect of these drugs on βTG concentrations.

Experiment i: The effect of Aspirin on plasma βTG concentrations

Methods:

Plasma βTG concentrations were measured in 9 healthy individuals aged 19-41 years (mean 29.1 years, SD 7.6 years) before, and 8 hours after, the ingestion of 300 mg Aspirin.

Results:

These are shown in Fig. 12. The concentrations were lower before Aspirin (median 22.0 ng/ml) than after (median 36.0 ng/ml) but this difference did not reach statistical significance using a paired Wilcoxon test ($p > 0.1$).

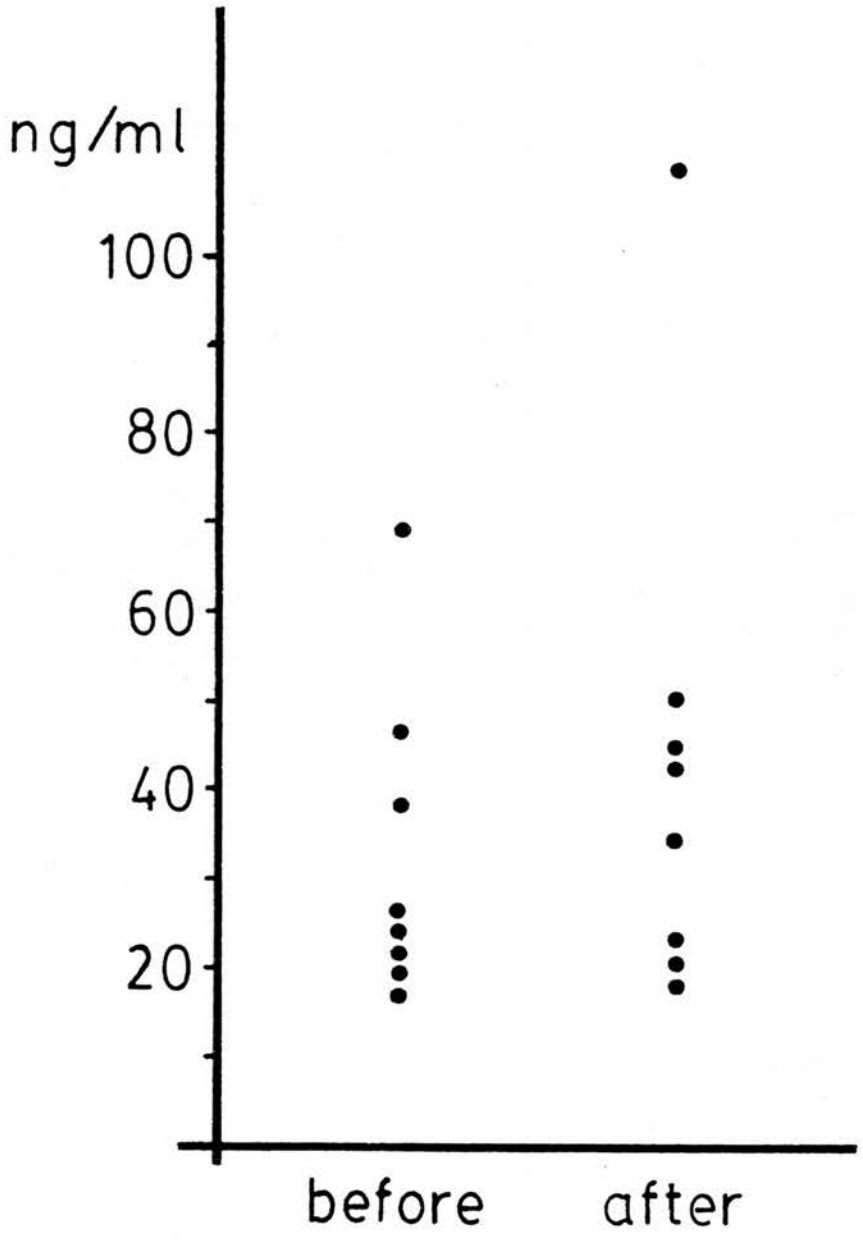

Fig. 12: Plasma βTG concentration before, and 8 hours after, 300 mg Aspirin in 8 healthy subjects.

Experiment ii: The effect of Aspirin on the syringe test

Methods:

Syringe tests were performed on 8 healthy individuals aged 20-41 years (mean 30.2 years, SD 6.9 years) before, and 8 hours after, the ingestion of 300 mg Aspirin.

Results:

Figure 13 demonstrates the results. The release times following Aspirin were shorter (median 5.5 mins) than before Aspirin (median 6.0 mins) and this did reach significance using a paired Wilcoxon test ($p < 0.05$). The basal concentrations rose slightly after Aspirin (median rising from 21.5 ng/ml to 29.0 ng/ml) but this was not statistically significant.

Experiment iii: The effect of intravenous Heparin on plasma and urine βTG concentrations

Methods:

Two healthy subjects aged 30 and 41 years, weighing 72 and 65 Kg respectively, were each given a single dose of 5,000 units of sodium Heparin intravenously. Plasma and urine samples were obtained at intervals before and up to 24 hours after injection. The plasma samples were obtained from the contralateral arm to that used for the injection of the Heparin and each sample was obtained from a point more distally on the limb. The samples were assayed for both PF_4 and βTG and the urine specimens were tested for blood with 'Labstix' (Ames).

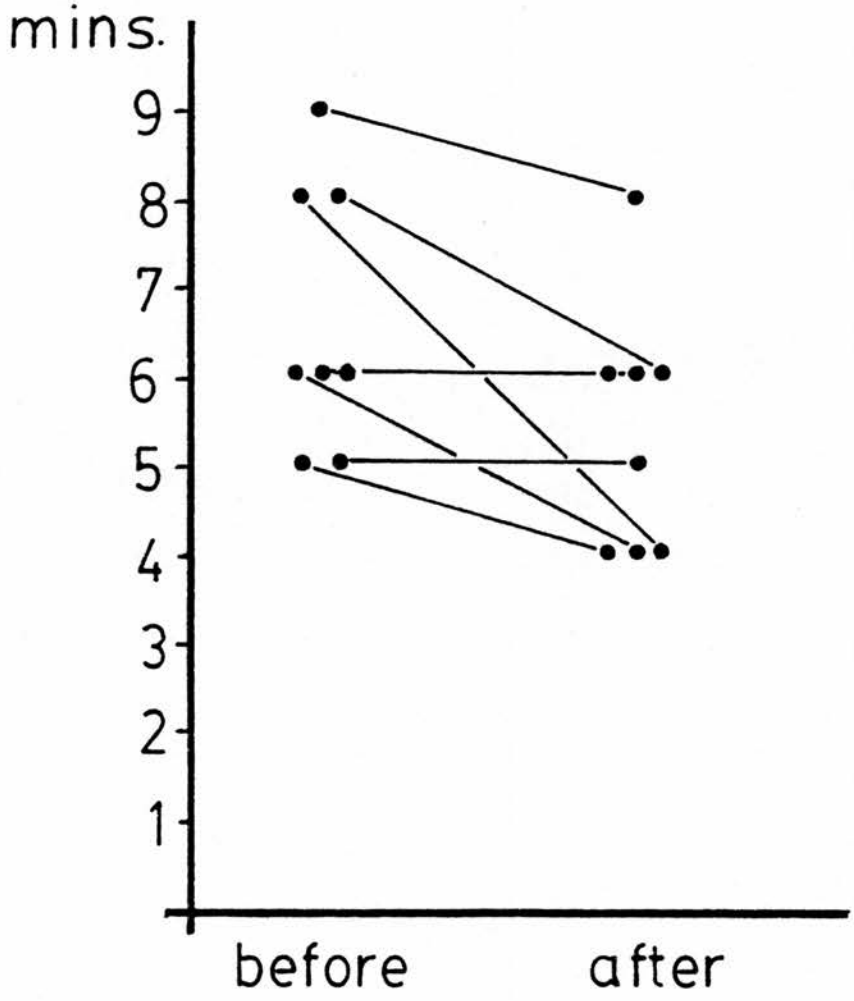

Fig. 13: Syringe test release times before, and 8 hours after, 300 mg Aspirin in 8 healthy subjects.

Results:

The effects of intravenous Heparin on subject 1 are shown in Fig. 14. Immediately after injection there was a massive release of PF_4 producing plasma concentrations > 240 ng/ml. This rise in the plasma concentration was associated with elevated urine concentrations. The plasma concentrations returned to normal within 1 hour and the urine concentrations within 2 hours. The plasma βTG concentrations, however, showed no significant rise immediately after injection but a variable response later. Subject 1 showed elevated concentrations (110 and 180 ng/ml) at 2 and 8 hours, with an intervening normal concentration (34 ng/ml) at 4 hours. Between 3 and 6 hours the urine concentration also reached the abnormal range (0.33 ng/ml) confirming that this was a genuine *in vivo* release. The plasma PF_4 concentrations were also elevated at 8 hours but not at 2 hours.

Subject 2 showed identical PF_4 release and urinary clearance. βTG concentrations also showed late rises but to a lesser extent than with subject 1 (87 ng/ml at 2 hours and 83 ng/ml at 4 hours). At no stage did subject 2 show abnormal urine concentrations (maximum - 0.19 ng/ml at 1 hour).

Experiment iv: The effect of subcutaneous Heparin on plasma and urine βTG concentrations

Methods:

Five healthy subjects aged 20-41 years (mean 27.5 years, SD 6.9 years) and weighing 61-76 Kg, were each given 10,000 units of

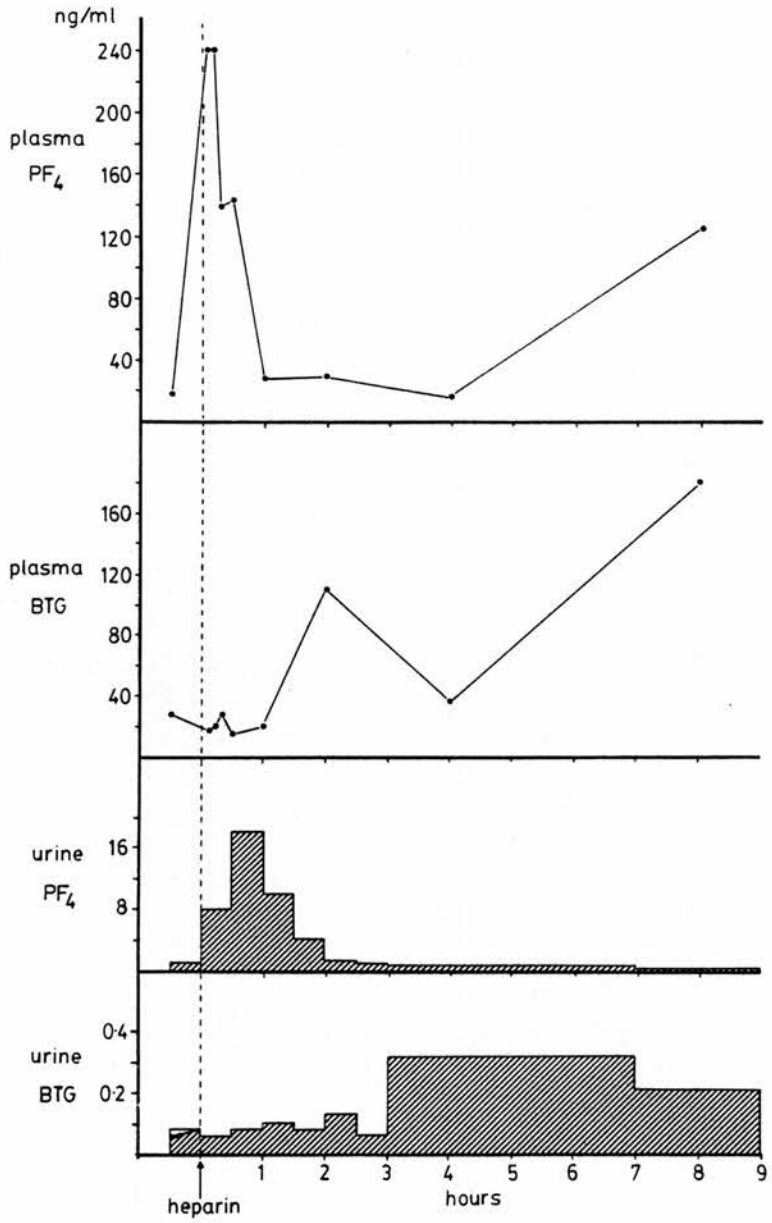

Fig. 14: Plasma and urine βTG and PF_4 concentrations before and after 5,000 units of heparin intravenously in a healthy subject.

sodium Heparin by deep subcutaneous injection into the abdominal wall. Plasma samples were obtained before, and for 24 hours after, injection. Urine samples were also obtained from each voiding during the same period.

Results:

Fig. 15 illustrates the plasma βTG concentrations in response to a subcutaneous injection of Heparin. Again there was no significant early release but, after 4 hours, several high concentrations were obtained. Every subject had at least one elevated plasma concentration after Heparin. The urine concentrations were all within the normal range except in subject 3 who showed elevated concentrations (> 1.0 ng/ml) throughout the 24 hour period after injection and subject 6 who showed one elevated concentration (0.33 ng/ml) immediately after injection. Neither of these individuals had evidence of haematuria on testing (Labstix).

Discussion

The dose of Aspirin required to produce a maximal effect on platelets appears to be quite critical. Burch *et al.*[25] have shown that 325 mg of Aspirin will produce an 89% inactivation of cyclo-oxygenase. This effect is at a maximum within 6 hours and persists for 2 days. Higher doses of Aspirin produce less inactivation. The dose used in this study (300 mg) and the time sampled (8-10 hours after ingestion) would appear to be ideal for detecting the maximal effect of Aspirin.

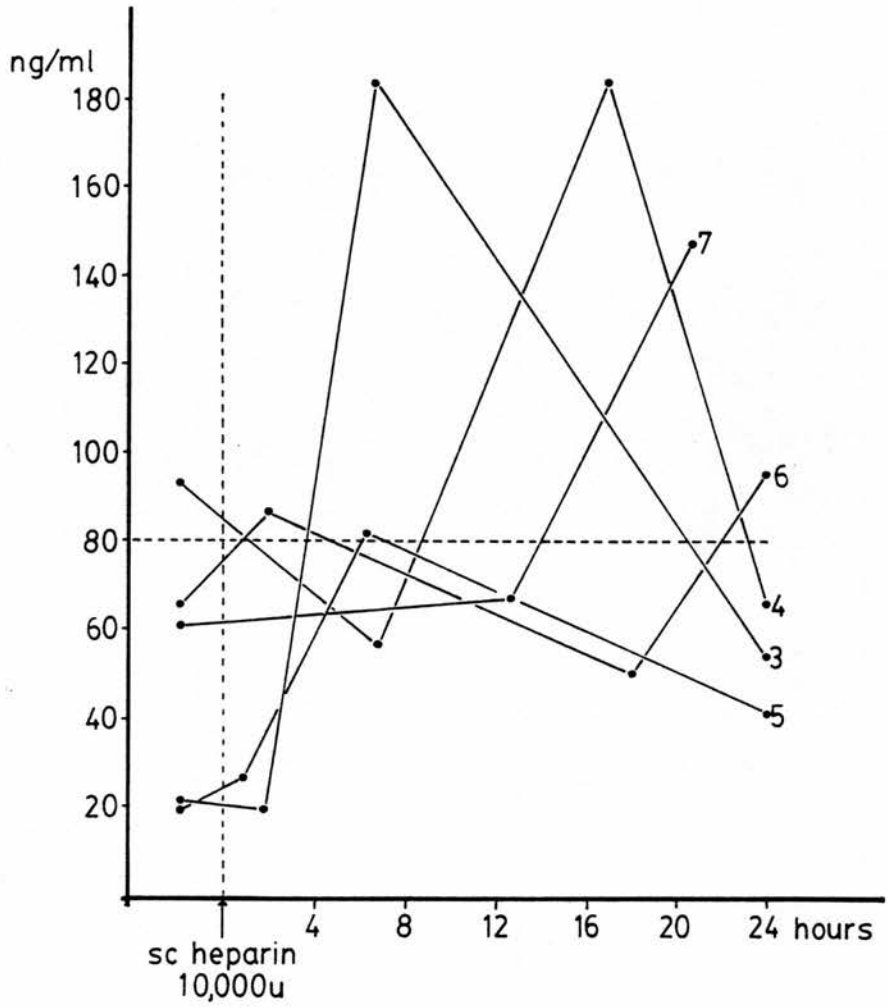

Fig. 15: Plasma βTG concentrations before and after 10,000 units of heparin given subcutaneously in 5 healthy subjects.

There are much published data on the disturbance of platelet function produced by Aspirin and some of it appears conflicting. Mills *et al.*[123] however, in a study of platelet function in patients with gastrointestinal bleeding, concluded that the platelets of some patients were much more sensitive to Aspirin than others. Quick[158] demonstrated that Aspirin prolonged the bleeding time and, in 1968, Evans *et al.*[57] showed that Aspirin would reduce platelet aggregation in response to thrombin provided the thrombin concentration was low. Release and aggregation occurred normally at higher thrombin concentrations, a fact confirmed by Zucker and Peterson[210] and Holmsen *et al.*[81] Evans *et al.*[57] showed that Aspirin inhibited the release of dense granule content in response to low concentrations of thrombin and Holmsen *et al.*[81] confirmed this but could demonstrate no effect on alpha granule release which they assessed by the release of acid hydrolases. Kaplan *et al.*[96] showed that, although Aspirin produced only a minimal reduction of acid hydrolase release, it did inhibit the release of PF_4, βTG, PDGF and fibrinogen.

These data lead us to expect lower βTG concentrations following Aspirin ingestion as there should be less platelet reactivity. However, as well as inhibiting the production of proaggregatory prostaglandin derivatives, Aspirin will also inhibit the production of the antiaggregatory vessel wall derivative PGI_2 (Prostacyclin). Although the duration of effect on PGI_2 production is probably less than on Thromboxane A_2 production,[188] it may be that the effect of Aspirin on the vessel wall is greater. This could explain the slightly higher plasma

βTG concentrations in the samples after Aspirin ingestion. If this is so, it could have considerable bearing on the dosage of Aspirin when used as a platelet inhibitor. A more frequent dose of Aspirin would have a relatively greater effect on PGI_2 production. If one considers the maximum effective dose (300 mg) and its duration of effect (the lifetime of the platelet) a dose of 300 mg only once or twice weekly may be the optimum for inhibition of aggregation.

The effect of Aspirin on the syringe test was also contrary to what was expected but followed the trend shown by Prowse *et al.*[157] The mean release time in their patients after Aspirin was reduced from 6.6 mins to 5.9 mins. The release reaction in the syringe test is mediated by thrombin generation and it is likely that the concentration of thrombin achieved exceeds that at which the Aspirin will still have an effect. However, there is at present no mechanism of action of Aspirin known which should make the release time in this situation shorter than normal.

Heparin is a naturally occurring anticoagulant which is used extensively in clinical practice. It is a potent inhibitor of coagulation, an activity thought to be due to its ability to bind to thrombin.[111] Its effect on platelets has also been extensively studied. As it has a potent antithrombin action it will obviously have an effect on thrombin induced platelet aggregation but it also affects cell membrane function[105] and may, therefore, have a more direct effect on platelet function.

Many papers describe an inhibitory effect on aggregation and on release. Besterman and Gillet[16] showed a reduction in the rate

and extent of collagen and adrenaline induced aggregation following 1,000-5,000 units intravenously and Eika[52] has described a platelet refractory state following Heparin. It has also been shown to inhibit platelet adherence to damaged and undamaged rabbit aorta *in vitro*[54] and in a study of pulmonary embolism in dogs. Thomas *et al.*[181] demonstrated that Heparin could prevent serotonin release from platelets *in vivo*. Mustard and Murphy[134] showed that subcutaneous Heparin in a dose of 5,000 units b.d. had no effect on platelet survival but, if the dose was increased to 8,000 units t.i.d., survival was prolonged. At low concentrations *in vitro* (25 u/ml), Heparin will inhibit thrombin induced aggregation but not that induced by ADP or adrenalin.[33] In higher doses (104 u/ml), however, it will also inhibit *in vitro* aggregation in response to collagen and adrenaline.

Rowsell *et al.*,[167] in a study in dogs, confirmed the inhibitory effect by showing that moderate doses of Heparin prolonged platelet survival but, as the dose was increased, the survival reached a peak and then fell off again. The dose at which this peak occurred varied considerably from dog to dog with a range of 9 to 381 units/Kg. In the subjects in this study the Heparin dose used was approximately 75 or 150 units/Kg and, therefore, some of the subjects may have received Heparin doses beyond the peak value.

Rowsell *et al.* also studied the lymph of dogs receiving very high doses of Heparin (600 units/Kg 8 hourly) and showed that, after 12-16 hours, the lymph contained red cells followed about 4 hours later by platelets. At the time when platelets appeared in the lymph, the blood platelets showed normal aggregation in response

to ADP, delayed aggregation in response to collagen and no aggregation with thrombin.

The passage of platelets into the lymph may be due to the failure of the maintenance of vascular integrity by the fibrin generation system and is likely to be associated with release as the platelets come into contact with extravascular collagen. Adherence of the platelets to extravascular collagen may explain the delay in platelets appearing in the lymph in relation to red cells. The βTG release I detected at various times after both iv and sc Heparin may be a manifestation of this platelet leakage from the circulation.

However, Zucker[211] has shown that Heparin will produce release from platelet rich plasma *in vitro,* presumably by a membrane effect, but whether this will occur *in vivo* is unknown. Enhanced primary waves of ADP and adrenaline induced aggregation have also been described in man after 10,000 units of Heparin iv.[182] These latter two effects may be contributing to the raised plasma βTG concentrations in this study. However, as detected by others,[167] the effect of Heparin varied considerably between individuals. Subjects 1 and 3 showed considerable release and prolonged elevation of urine concentrations in contrast to the normal concentrations detected in most of the others.

The effect of Heparin on PF_4 release is in marked contrast to its effect on βTG release. There is an immediate and rapid rise in PF_4 concentration following Heparin which has returned to normal in 1 hour. This suggests that PF_4 is stored in a different

subpopulation of these granules from βTG or that there is another source of PF_4 such as vascular endothelium. PF_4 has an ability to neutralise Heparin by binding to it and thus release in response to Heparin is likely to be a form of defence mechanism. The Heparin neutralising activity of platelets has been recognised since 1948 when Conley *et al.*[34] showed that the concentration of Heparin required to inhibit or delay coagulation is directly related to the platelet count. In subject 1 βTG release occurred at 2 hours but this was not associated with PF_4 release. At 8 hours, however, the βTG and PF_4 concentrations rose in parallel. This suggests that it takes up to 8 hours for the PF_4 stores to be replenished. The release of PF_4 in response to Heparin may be of significance in the development of relative resistance to Heparin by some patients on Heparin therapy.

6. CLEARANCE OF PLASMA BETA-THROMBOGLOBULIN AND ITS URINARY EXCRETION PATTERN FOLLOWING AUTOLOGOUS SERUM INFUSION

The studies in part 2 did not demonstrate any relationship between plasma and urine concentrations of βTG in normal subjects. In that particular study the ranges of concentrations of βTG in plasma and urine were narrow and in that situation demonstration of mathematical correlation becomes more difficult. However, it may be possible to demonstrate a correlation when the concentration ranges are greater, such as in patients with thromboembolic disease.

If the plasma βTG concentration is artificially increased by infusing βTG, it is possible to assess the rate at which it is cleared from the plasma and also the rate at which it appears in the urine. It should also be possible to calculate the proportion of the infused dose which appears in the urine.

In serum and in the supernatant of platelet concentrate, βTG is found in high concentrations. Serum is technically easier to prepare and this was selected. Platelet infusion was employed in one subject who had undergone platelet and plasmaphoresis for another purpose.

Methods:

One unit of blood was withdrawn using standard aseptic technique into a plastic transfusion bag without anticoagulant. This was allowed to clot for 12 hours and the serum was drawn off into a second plastic bag. It was then stored for 36 hours at 4^0C to remove the thrombin by binding to the plastic.

The serum was infused into an ante cubital vein over 10 mins and plasma samples for βTG assay were obtained before re-infusion, immediately after re-infusion, every 10 mins for half an hour and every 30 mins for 3 hours. Urine specimens were obtained every half hour up to 4 hours after infusion.

Subject 1 underwent two serum and one platelet concentrate infusions (experiments i, ii and iii) and subject 2 received only two serum infusions (experiments iv and v).

Results:

All the infusion studies showed similar patterns of βTG plasma clearance and urinary excretion. The results of the second infusion study in subject 1 are shown in Fig. 16 and the plasma clearance is shown plotted semi-logarithmically in Fig. 17.

The data for all the infusion studies are summarised in Tables 7 and 8. The half hourly urinary βTG excretion rate in experiment iv is shown in Fig. 18.

The dose of βTG varied between studies with the largest dose being derived from the platelet concentrate as this gave the highest plasma concentration and total urinary βTG output.

All plasma and urine concentrations were within the normal range before each infusion and were again normal on completion of the 3 hour post-infusion period.

Figures 16 and 17 show that, immediately following infusion, βTG is rapidly cleared from the plasma with a half life varying from 9 to 23 mins. After 30-40 mins a phase of slower clearance

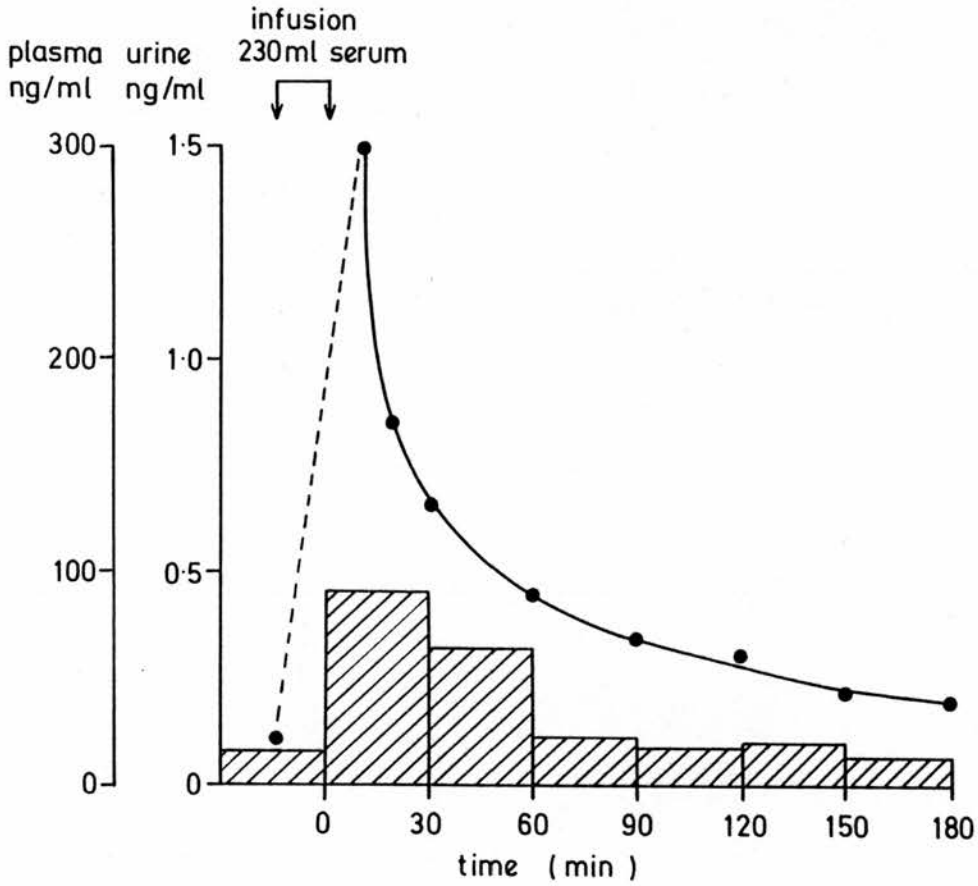

Fig. 16: Clearance of plasma βTG in a healthy subject following autologous serum infusion.

(Urine concentration cross hatched.)

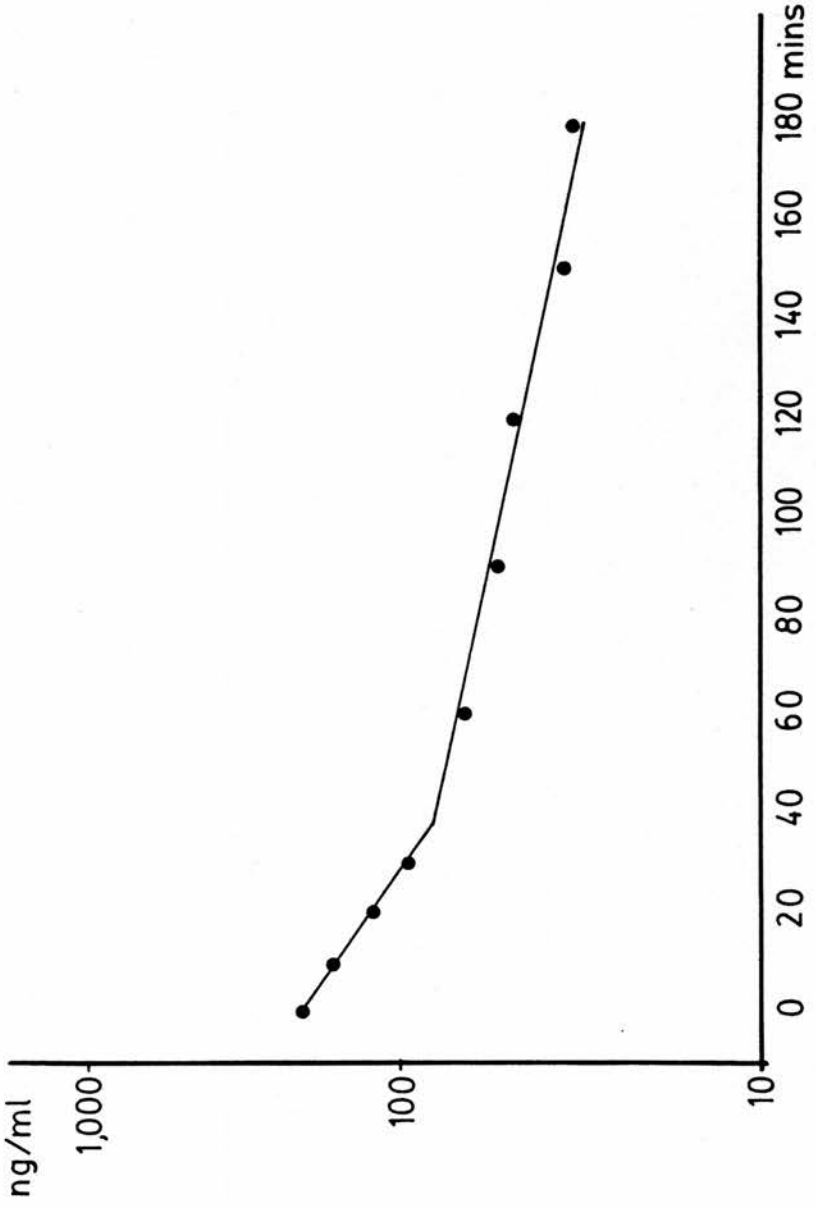

Fig. 17: Semi-logarithmic plot of plasma βTG clearance.

TABLE 7: PLASMA βTG CONCENTRATIONS FOLLOWING 4 AUTOLOGOUS SERUM INFUSIONS AND 1 AUTOLOGOUS PLATELET INFUSION IN 2 SUBJECTS. (r represents the correlation between the log plasma concentration and time.)

Subject	Experiment	Pre-infusion concentration (ng/ml)	Peak concentration (ng/ml)	Plasma half life (mins)	r
I	1	19	235	75	0.92
	2	18	297	89	0.98
	3	28	1915	86	0.95
II	4	22	174	101	0.91
	5	20	466	64	0.99

TABLE 8: URINARY βTG CONCENTRATIONS AND EXCRETION FOLLOWING 4 AUTOLOGOUS SERUM INFUSIONS AND 1 AUTOLOGOUS PLATELET INFUSION IN 2 HEALTHY SUBJECTS

Experiment	Peak concentration (ng/ml)	Mean hourly urine output (ml/hr)	Peak excretion rate (ng/hr)	Total excretion (ng)
1	0.95	22	14.2	17.6
2	0.46	53	10.2	13.1
3	1.0	35	58.0	68.7
4	0.15	79	22.4	18.8
5	0.19	182	27.2	23.9

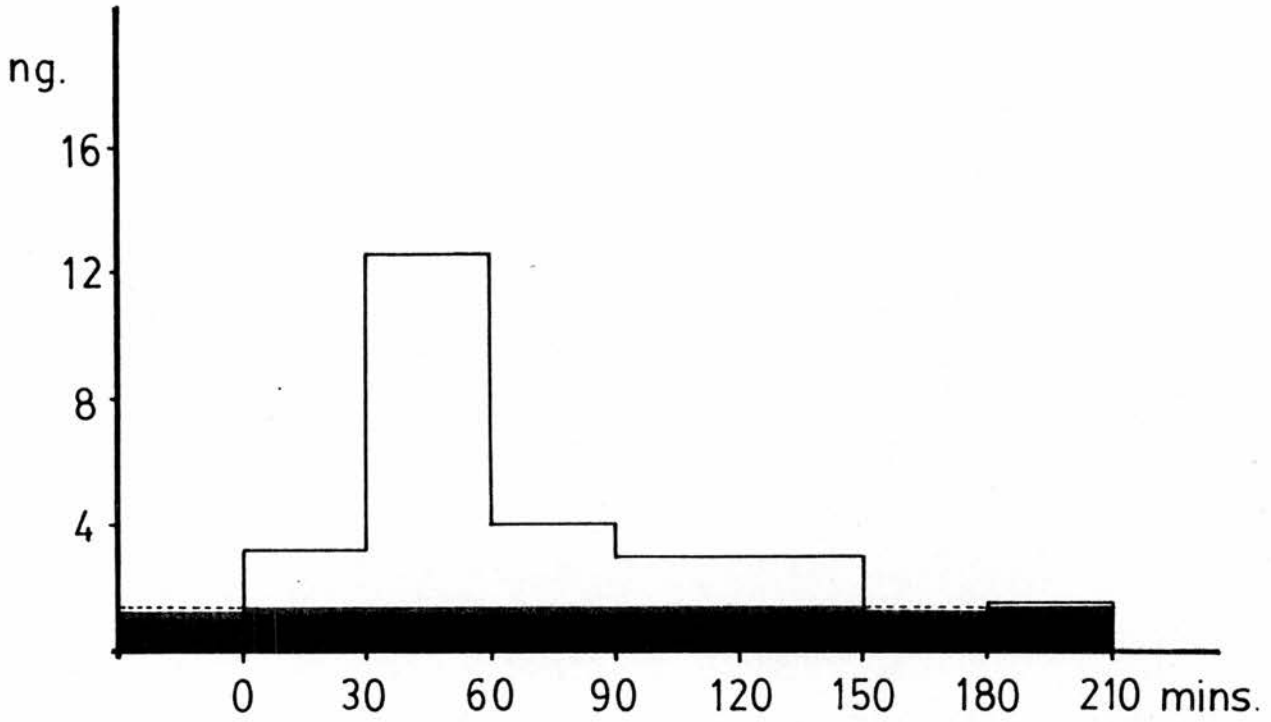

Fig. 18: Urinary βTG excretion (ng/½ hr) following autologous serum infusion. (Shaded area represents calculated basal excretion rate. Serum infusion completed at time 0.)

commences. In subject I the rates of the plasma βTG clearance in this second phase correlated quite well between studies with half lives varying from 75 to 89 mins (mean 83 mins). In subject II the clearance patterns were less consistent (64 and 101 mins) but the mean value (82.5 mins) correlated well with the mean value for subject I.

The duration of the elevated plasma βTG concentration correlated with the maximum plasma concentration and is, therefore, related to total βTG dose.

The correlation between the log of the plasma βTG concentration and the time after infusion was good in all the studies ($r = 0.91-0.99$) and, therefore, the clearance is truly exponential.

The urine excretion of βTG tended to follow the plasma clearance. This was apparent in both the urine concentrations and urinary βTG excretion rates in subject I, but in subject II the urinary concentrations never reached the abnormal range and, therefore, there was little correlation between the urine concentration and the plasma clearance. However, if the βTG excretion rate is shown in ng/½ hour (Fig. 18), the close correlation with the plasma clearance can be demonstrated.

The total βTG excretion is a measure of the amount of βTG excreted in excess of the normal excretion. The normal βTG excretion rate was established by taking the mean of the excretion rates for the half hour periods before and after the post-infusion collections. By subtracting this value from the total excretion for each half hour an approximation of the excess βTG excreted

could be calculated. This is represented by the area above the shaded area in Fig. 18.

The total βTG excretion is related to the peak excretion rate, the maximum urine concentration and, in subject I, to the duration of elevated urinary concentration. The peak βTG excretion rate always occurred within the first hour of infusion.

In two studies (1 and 4) accurate measurements were available for the concentration of βTG in the infused serum (8,538 and 10,879 ng/ml). It was, therefore, possible to calculate that approximately 0.002% of the infused βTG in each of these studies appeared in the urine.

Discussion

The platelet concentrate produced the highest dose of βTG. As the concentrate was derived from two units of blood, twice the number of platelets were involved in the preparation of the platelet concentrate compared to the serum. Another contributory factor is the time of platelet release in relation to the separation of the serum or platelets. In the preparation of serum, the platelets release before the serum is separated and, therefore, much of the βTG is lost in the clot that is separated off. In the case of the platelet concentrate, the platelets were separated in a cooled centrifuge immediately after collection and, therefore, most of the release occurs after separation. Consequently much less βTG is lost.

The early very rapid clearance observed within the first half hour of completion of the infusion is typical of smaller protein

molecules which do not remain solely in the plasma space. This represents the distribution of the protein into extravascular spaces combined with some excretion and metabolism. After about 30-40 mins this process reaches equilibrium and the subsequent slower clearance represents the excretion and metabolism of the protein. The half life of this phase for βTG appears to be about 83 mins. This is significantly longer than the half life of PF_4[40] and of FPA[141] which may be an advantage when used as a diagnostic test. It may be long enough to enable one to detect short lived episodes of platelet release but not so long as to interfere with the detection of recurrent episodes of release over a period of hours. However, from a clinical standpoint, this half life may be too short to enable one to identify episodic release by once daily sampling. The urinary excretion of βTG seems to follow the plasma clearance fairly closely. In subject I this can be detected on the urinary concentration alone but the urine flow rate tended to be low in all three studies. Subject II had no elevated urinary concentration at all and it was only possible to show the correlation by charting the actual βTG excretion rate. This is consistent with the finding in section 2 of this chapter which demonstrated that normal individuals can dilute their urinary βTG concentrations with minimal diuresis. Subject II had urine outputs two to three times those of subject I and this is the reason for the discrepancies in urinary concentrations. The actual βTG excretion rates in subject II showed the same patterns as subject I.

The proportion of infused βTG which appears in the urine is tiny (one fifty thousandth of the infused dose), and therefore it

forms a very minor route of βTG clearance. However, it does reflect quite closely the plasma concentrations of βTG and, therefore, has potential value in the assessment of platelet release in clinical and experimental situations.

7. COMPARISON OF PLASMA BETA-THROMBOGLOBULIN CONCENTRATIONS MEASURED BY THE ORIGINAL TECHNIQUE AND BY THE RADIOCHEMICAL KIT

When this study first commenced the Radiochemical Centre at Amersham were developing the βTG radioimmunoassay kit and for a short period samples from the study were assayed both by the MRC Radioimmunoassay Laboratory in Edinburgh and by the Radiochemical Centre Kit.

Methods:

A total of 174 samples were drawn from 38 patients in the thromboembolism and post-operative surveys. After plasma separation half of each sample was sent to the Radiochemical Centre at Amersham and assayed there using their RIA kit. The other half was processed in the normal fashion in Edinburgh.

Results:

Table 9 and Fig. 19 summarise the results. The correlation between the two methods was significant ($p < 0.01$). The concentrations measured by the MRC assay are approximately twice those measured by the RIA kit. The arithmetical linear regression [MRC concentration = 27 + 0.123 x RIA kit concentration] was markedly distorted by the poorly correlating high values and, therefore, a regression line to Fig. 19 has been added on a purely geometrical basis. This suggests that an upper limit of normal of 80 ng/ml on the MRC assay corresponds to about 40 ng/ml on the RIA kit assay. On this basis, 52 of the samples had elevated concentrations on both assays; 111 of the samples had normal

TABLE 9: CORRELATION BETWEEN MRC RADIOIMMUNOASSAY LABORATORY PLASMA βTG CONCENTRATIONS AND RADIOCHEMICAL CENTRE KIT PLASMA βTG CONCENTRATIONS

Group	Range (ng/ml)	Median (ng/ml)	Mean (ng/ml)	SD	No. > upper limit	
					Not correlating	Correlating
MRC	15-1342	51	98.3	172.8	5	52
AM	2-340	26	39.2	41.0	6	52

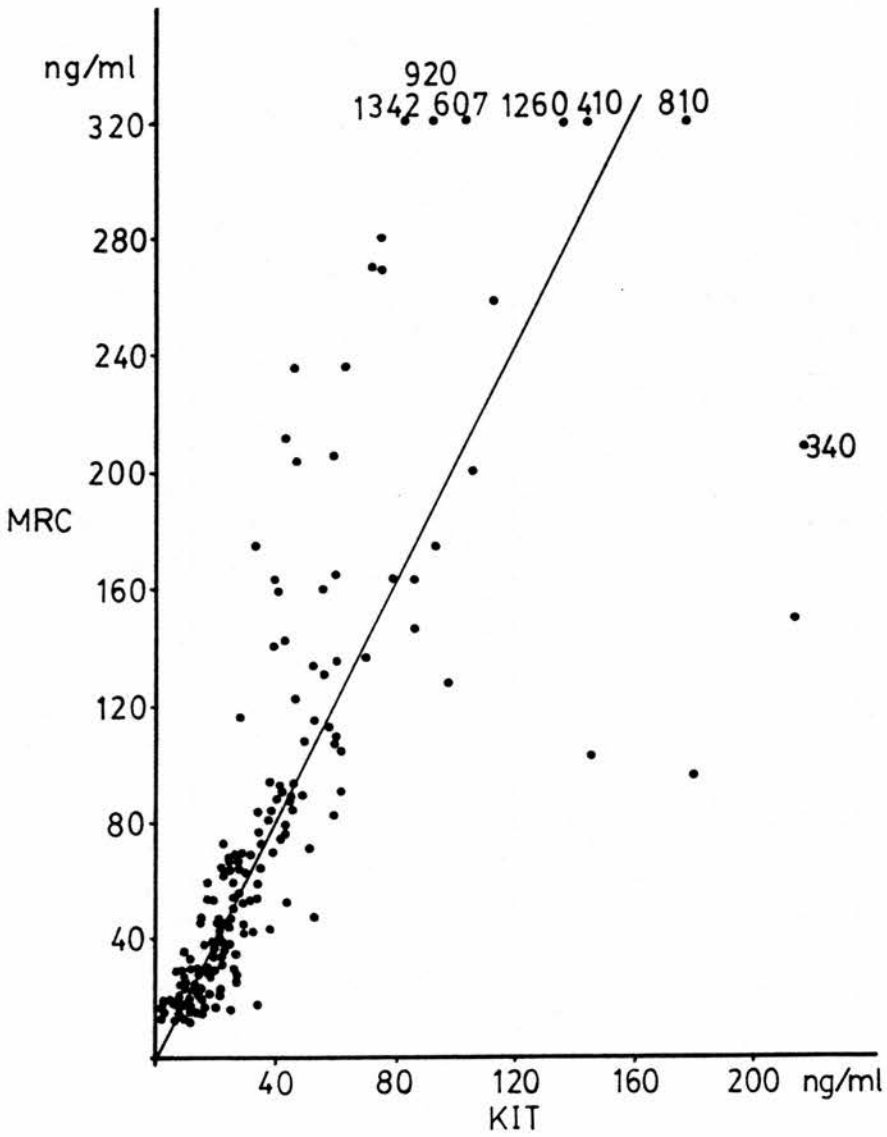

Fig. 19: MRC plasma βTG concentrations v Radiochemical Centre RIA Kit plasma βTG concentrations on 174 samples.

concentrations on both assays; 5 had elevated concentrations on the MRC assay alone and 6 had elevated concentrations on the RIA kit assay alone. Only 9 samples had higher concentrations on the RIA kit assay than on the MRC assay.

Discussion

The Radiochemical Centre kit is consistently less sensitive than the MRC assay and this serves to reinforce the point that all laboratories should establish their own normal range with a new diagnostic test before using it clinically.

Our results are consistent with those quoted in the Radiochemical Centre RIA Kit handbook where combined data from four centres showed that 95% of normal subjects had concentrations less than 52 ng/ml.

The good correlation between the two assays suggests that they are measuring the same plasma constituent.

CHAPTER II

STUDIES IN PATIENTS WITH VENOUS THROMBOEMBOLIC DISEASE

Plate aggregation has been known to be an integral part of the formation of thrombi in the venous circulation since the description of the histological appearance of thrombi by Welch.[197] Although the relative numbers of platelets in venous thrombi are lower than in arterial thrombi, they are still present in very much higher proportions than in blood.[150] There is some evidence that the aggregation of platelets in the valve cusps may be the earliest event in the development of deep vein thrombosis.[192] The subsequent propagated thrombus has a widely varied content of platelets typified by the classical laminated thrombus which is composed of alternating bands of platelet rich and platelet poor thrombus.[70] As platelet aggregation is associated with βTG release, elevated βTG concentrations may, therefore, be the earliest manifestation of a developing venous thrombus.

Further support for this theory came from Ludlam *et al.*[107] who studied patients with calf pain undergoing ascending phlebography. In patients with positive phlebograms the plasma βTG concentrations were all elevated in contrast to the normal concentrations in a further 8 patients with negative phlebograms.

An ideal diagnostic test for venous thrombosis is one which will accurately define those patients with DVT and those without (i.e. it should be sensitive and specific). This preliminary study suggested that the plasma βTG concentration fulfils this criterion.

It is also a simple, inexpensive and rapidly performed test which may be readily repeated any number of times.

Experiment i: Plasma and urinary βTG concentrations in venous thromboembolism

Methods:

Three groups of patients were studied.

Group 1: This group, which acted as controls, was composed of 18 patients presenting with leg pain whose symptoms were due to conditions other than venous or arterial thrombotic disease. The details of these patients are shown in Table 10. There were 8 males and 10 females aged 36-74 years (mean 60.1, SD 9.9 years) and, of these, 8 had had symptoms for < 3 days and 10 had had symptoms for > 3 days. Only 7 patients had phlebograms. The other patients had no clinical evidence of thrombotic venous or arterial disease but did have definite clinical and/or radiographic evidence to support an alternative diagnosis. Plasma and urine samples were obtained from all patients at the time of presentation and in 9 patients further specimens were obtained for up to 72 hours afterwards.

Group 2: This comprised 46 patients presenting with leg pain and swelling or chest pain and dyspnoea in whom a diagnosis of venous thromboembolism could be substantiated. The details of these patients are shown in Table 11. There were 17 males and 29 females aged 26-81 years (mean 58.4, SD 12.3 years). In 39 the diagnosis was confirmed by phlebography, in 3 by Doppler ultrasound blood flow

TABLE 10: THROMBOEMBOLISM STUDY CONTROL PATIENT DATA AND PLASMA βTG CONCENTRATIONS

No.	Age	Sex	Diagnosis	Phlebo-gram	Symptoms days	Plasma βTG ng/ml Day 1	Plasma βTG ng/ml Range
1	68	M	Varicose veins	-	> 3	55	55-100
2	48	F	Varicose veins	-	> 3	245	-
3	65	F	Neuropathy	-	> 3	1725	-
4	68	M	Neuropathy	-	> 3	76	-
5	74	F	Osteoarthritis	-	> 3	1012	-
6	61	M	Spondylosis	-	> 3	110	-
7	54	M	Spondylosis	-	> 3	45	-
8	74	M	Spondylosis	-	> 3	24	-
9	60	M	Spondylosis	-	> 3	44	-
10	68	F	?	-ve	> 3	22	20-139
11	58	F	?	-ve	< 3	47	-
12	54	F	Cellulitis	-	< 3	118	70-118
13	45	F	Baker's cyst	-ve	< 3	128	-
14	61	F	Sup. phlebitis	-ve	< 3	99	-
15	63	M	Haematoma	-	< 3	49	-
16	36	F	Post-operative	-ve	< 3	45	45-202
17	65	M	Post-operative	-ve	< 3	222	-
18	59	F	Post-operative	-ve	< 3	56	-

TABLE 11: THROMBOEMBOLISM STUDY PATIENT DATA AND PLASMA BETA-THROMBOGLOBULIN CONCENTRATIONS

No.	Age	Sex	Aetiology	Symps. days	Test	Site	Lung scan	Plasma βTG ng/ml Day 1	Range
19	60	M	Post-op	< 1	BAV	Nil	+ve	50	42-1410
20	55	F	Post-op	1	BFV	LFICv	-ve	47	47-115
21	56	M	Post-op	1	BAV	BCF	-ve	48	48-178
22	45	F	Post-op	> 3	BAV	RCF	+ve	112	17-211
23	51	F	Post-op	> 3	BAV	LC	-ve	31	14-39
24	37	M	Post-op	1	BAV	Nil	+ve	17	17-20
25	68	M	Spont.	> 3	BFV	LFI	-	61	16-109
26	43	F	Post-op	1	BAV IFT	BC	+ve	93	93-700
27	69	F	Malig.	> 3	BFV	LFI	-	563	-
28	41	F	Spont.	1	LFV RAV	LFI	-ve	41	41-106
29	55	F	Post-op	> 3	BFV IFT	RC	+ve	2600	94-2600
30	55	F	Spont.	> 3	BAV	BC	-ve	47	33-98
31	33	F	Spont.	1	BAV	RCFI	+ve	777	25-777
32	76	F	Spont.	> 3	LAV RFV	LCFI	-ve	93	24-111
33	67	F	Spont.	> 3	BAV	RFI	+ve	90	30-90
34	75	F	Post-op	2	BFV IFT	BC	+ve	220	99-220
35	58	M	Inflam.	> 3	BFV	RCFI	+ve	15	15-32
36	50	M	Inflam.	2	RAV	RFI	-ve	25	25-2549
37	57	M	Malig.	1	BFV	LFI	+ve	698	67-698
38	59	F	Malig.	1	BFV	LFI	+ve	258	29-258
39	26	F	Trauma	> 3	BFV	RFI	+ve	105	103-105
40	76	F	Post-op	1	BFV	RFI	-ve	66	38-66
41	60	M	Post-op	1	BAV	BC	-ve	90	51-90
42	72	F	Spont.	> 3	BAV BFV	LCFI	-ve	62	52-113
43	73	F	Inflam.	> 3	BAV	LCFI	-ve	80	23-166
44	68	F	Spont.	1	BAV	BC	-	180	25-731
45	65	F	Post-op	> 3	BFV	LFI	-ve	64	38-576
46	66	F	Inflam.	1	BFV	LFI	-ve	91	37-860
47	59	F	Trauma	> 3	BAV	LC	-	16	-
48	65	F	Malig.	1	BFV	LFI	-	338	338-400
49	73	F	Inflam.	2	BFV	LFI	+ve	135	49-135
50	52	M	Post op	> 3	BAV	LFI	+ve	94	50-2686
51	58	M	Trauma	2	BAV	LC	-	41	-
52	57	M	Post-op	3	BAV	RCF	+ve	106	-
53	43	F	Post-op	1	BFV	LFI	+ve	89	78-300
54	50	F	Post-op	1	IFT	LF	-	4000	-
55	63	F	Post-op	1	US	LF	-	195	117-195
56	56	M	Post-op	2	US	LF	-	55	55-103
57	76	M	Malig.	2	IFT	LC	-ve	86	72-86
58	61	F	Spont.	1	BAV	RC	-	99	-
59	46	M	Post-op	3	BAV	LC	-	69	23-69
60	65	M	Post-op	2	BAV	RC	-	201	-
61	81	M	Inflam.	3	US	LFI	-ve	108	94-142
62	61	F	Post-op	2	BAV	RC	-	163	-
63	42	M	Post-op	3	BFV	LFI	+ve	187	160-360
64	63	F	Post-op	1	LFV	LFI	-ve	450	310-450

Abbreviations:
B-bilateral, R-right, L-left, AV-ascending phlebogram, FV-femoral phlebogram, IFT-^{125}Iodine fibrinogen uptake test, US-Doppler ultrasound, C-calf, F-femoral, I-iliac, Cv-cava.

detection and in 2 by the ^{125}IFT. Thirty-two patients also had chest radiographs and perfusion lung scans using ^{99m}Tc labelled microspheres of human albumin and in 17 the diagnosis of PE was substantiated. Two patients had evidence of PE on lung scans but negative ascending phlebograms.

The 46 patients were then grouped according to possible aetiological factors. In 10 patients no apparent associated factor was identified and these have been deemed 'spontaneous'. None of the patients was on an oral contraceptive. Five patients with disseminated malignant disease developed DVT; in 5 the thrombus was associated with an inflammatory illness such as proctitis, cholecystitis or pneumonia and in 3 it followed trauma; 23 patients developed DVT following surgery: within 1-14 days of operation in 16 and between 14 and 32 days in 7. There were almost twice as many females as males and this was most evident in the 'spontaneous' group where males were outnumbered by a factor of 4. In 65% of patients, symptoms had been present for < 3 days; in 30% the thrombus was confined to the calf; in 5% no residual thrombus was identified in the legs; and in 65% the iliofemoral segment was involved.

Just over half (53%) of the 32 patients who had lung scans showed evidence of PE and the incidence of positive lung scans was highest (73%) in the early post-operative group.

There was no significant difference between the groups in any of the aetiological factors studied.

All patients had plasma samples for βTG assay taken at the time of presentation and in 38 of the 41 patients undergoing phlebography the first sample was obtained before the phlebogram. Sampling was repeated daily for up to 7 days.

Seven patients received no active treatment; 2 were given thrombolytic therapy and the remaining 37 were anticoagulated by continuous intravenous infusion of heparin. The heparin was given in a dose sufficient to maintain the thrombin time at 3-4 x the control.

When group 1 was compared with group 2 there was no significant difference between the groups in any of the population statistics, i.e. they are statistically comparable.

Group 3: This was a subgroup of group 2 and consisted of patients nos. 41 and 45-64 in whom urine samples as well as plasma samples were obtained. There were 9 males and 12 females aged 42-81 years (mean 58.2, SD 12.4 years). The diagnosis was confirmed by phlebography in 16, by Doppler ultrasound in 3 and by ^{125}IFT in 2. Eleven patients also had chest radiographs and lung scans, of which 5 were positive.

Four patients developed DVT > 2 weeks after operation and 10 developed it within 2 weeks; in 2 it occurred during an inflammatory illness; in 2 it followed trauma; in 2 it developed in patients with disseminated malignancy and in 1 it developed 'spontaneously'.

In 57% of patients the thrombus involved the iliofemoral segment but in 43% it was confined to the calf.

Group 3 did not differ significantly from group 1 in population statistics.

Results:

A. *Plasma βTG*

The results in all these groups are shown in Tables 10 and 11 related to the investigative findings.

1. *Plasma βTG on day of presentation.* These data are shown diagrammatically in Fig. 20 and summarised in Table 12. In 29 (63%) of patients presenting with venous thromboembolism, the plasma βTG concentration was in the abnormal range (>80 ng/ml) but in only 19 (41%) did it exceed 100 ng/ml.

In group 1 two specimens have been excluded. They showed concentrations > 1,000 ng/ml and were processed in the same batch as two other specimens showing unexpectedly high concentrations. The discrepancy has been attributed to release during faulty processing of that particular batch of samples. Of the remaining 16 specimens, 7 (44%) had concentrations greater than normal.

There was no significant difference in incidence of abnormal concentrations between groups 1 and 2 [$\chi^2 = 1.11$, $p = 0.29$], nor was there any difference in the absolute concentrations ($p > 0.1$, Wilcoxon). In the 33 patients who had lung scans there was a significantly greater incidence of elevated concentrations in those with positive scans (82%) than in those with negative scans (38%) [$\chi^2 = 5.24$, $p = 0.022$]. The absolute concentrations in those with DVT and PE (median 106 ng/ml) were also significantly higher than in those with DVT alone (median 81 ng/ml) ($p < 0.01$, Wilcoxon).

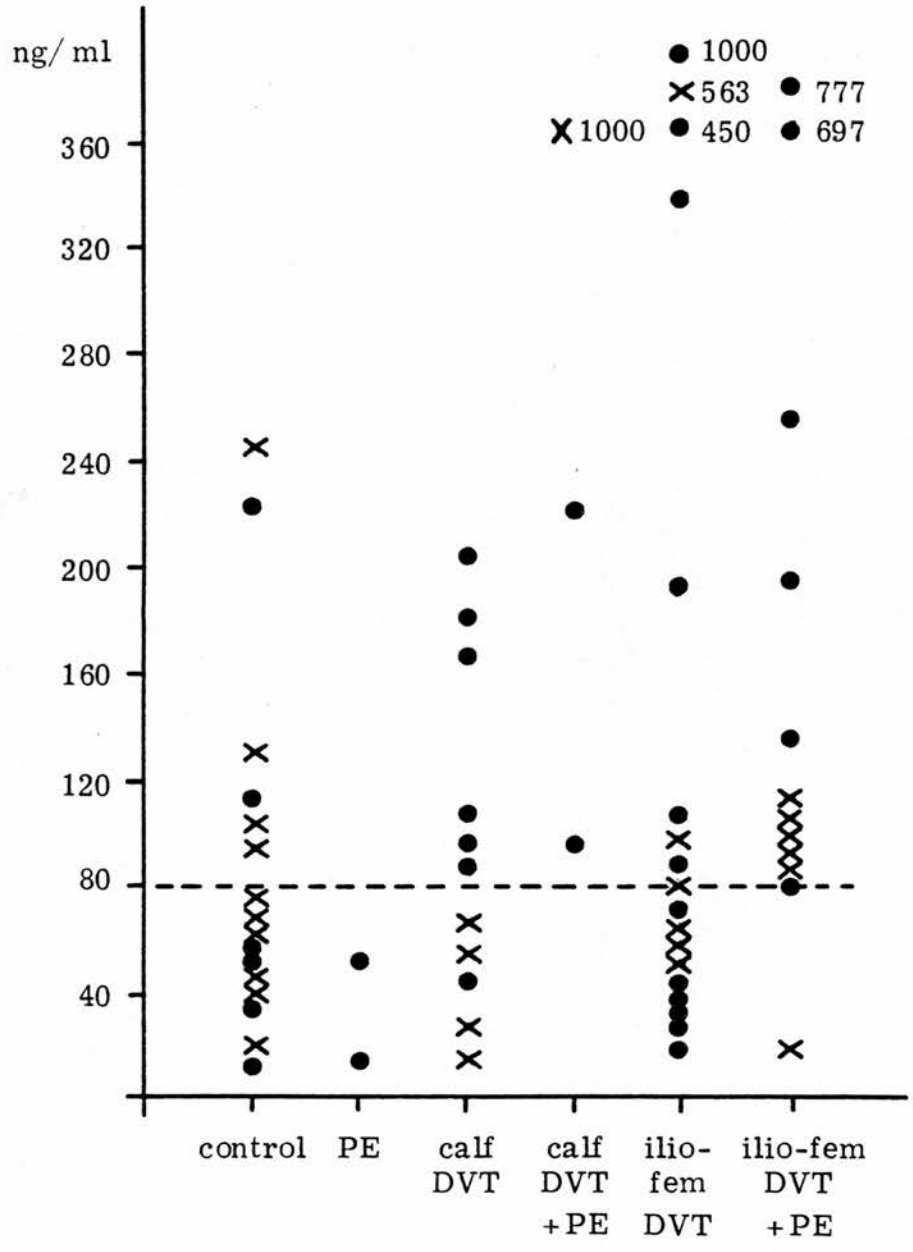

Fig. 20: Plasma βTG concentrations in patients with venous thromboembolism and in control subjects on day of presentation. (Crosses represent those with symptoms for > 3 days; dots represent those with < 3 days symptoms.)

TABLE 12: PLASMA βTG CONCENTRATIONS IN THROMBOEMBOLISM PATIENTS AND IN CONTROL SUBJECTS ON DAY OF PRESENTATION (DAY 1) AND ON SUBSEQUENT 3 DAYS

Group	DAY 1			DAYS 1-3		
	No. of specimens	Range (ng/ml)	Median (ng/ml)	No. of specimens	Range (ng/ml)	Median (ng/ml)
Control	16	22-245	66	23	20-245	76
DVT	46	15-1,000	92	114	15-1,410	90

There was no relationship between the duration of symptoms and the incidence of abnormal βTG concentrations. Of the 27, 18 (67%) with symptoms for less than 3 days and 11 of the 19 (58%) with symptoms for more than 3 days had elevated concentrations [$\chi^2 = 0.368$, $p = 0.54$]. There was also no difference in absolute concentrations ($p > 0.1$, Wilcoxon).

Those with DVT involving the iliofemoral segment were no more likely to have elevated concentrations (20/30) than those in whom the DVT was confined to the calf (9/14) [$\chi^2 = 0.024$, $p = 0.82$].

Phlebography did not appear to affect plasma βTG levels as the 3 patients whose initial samples were obtained after phlebography showed the same distribution of initial concentrations as those whose samples were obtained before phlebography. There was also no significant difference in βTG concentrations between the days before and after phlebography ($p > 0.1$, Wilcoxon).

2. *Follow-up and effect of therapy*. Plasma βTG concentrations followed for up to 3 days in 5 patients prior to commencing heparin therapy are summarised in Fig. 21. Four showed persistently elevated concentrations despite quite large fluctuations and the fifth showed normal concentrations.

In 28 patients results are available for one day prior to heparinisation and for 2-6 days after commencing heparin therapy. These data are summarised in Fig. 22. The median has been used as a summary statistic because of the skewed distribution. After commencing heparin therapy, 7 patients showed rising concentrations but in 21 the concentrations either fell or remained unchanged.

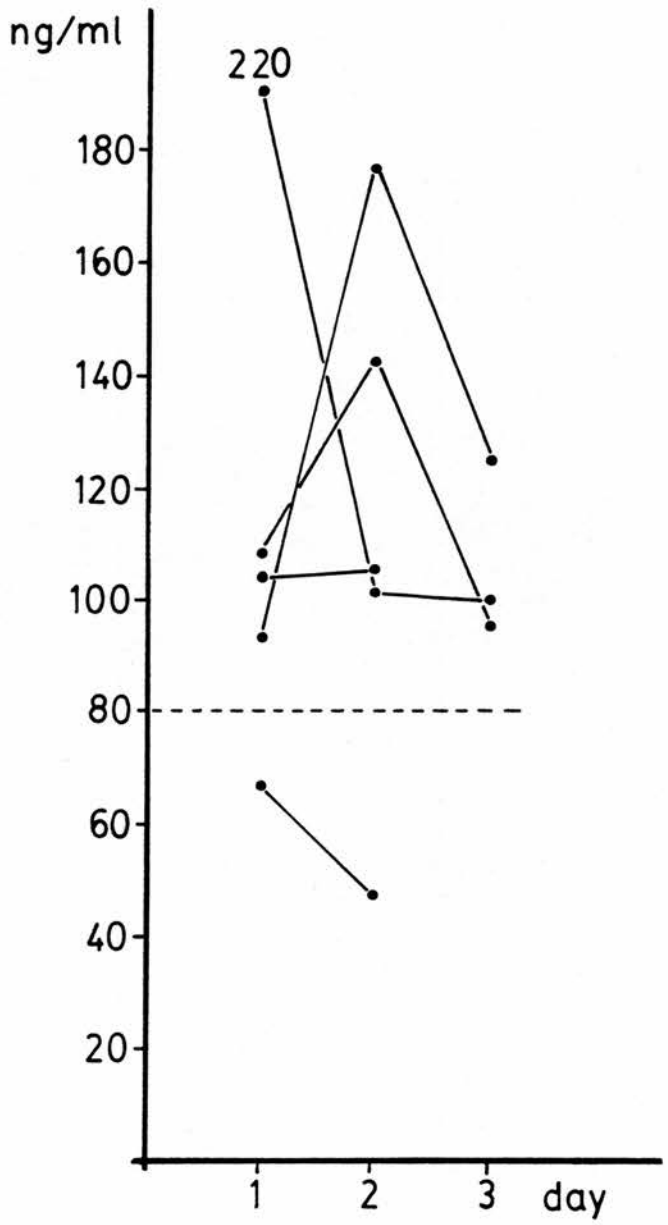

Fig. 21: Plasma βTG concentrations in 5 patients with venous thromboembolism prior to heparin therapy.

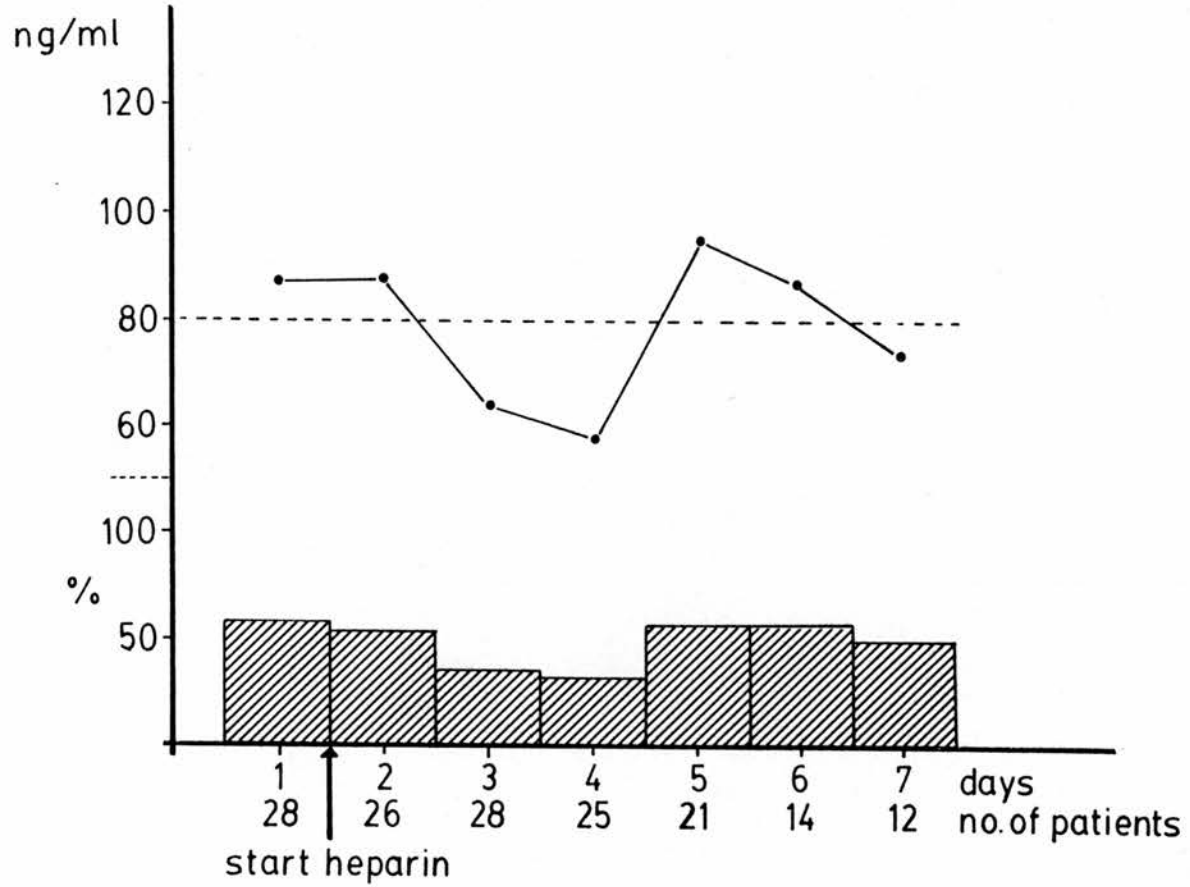

Fig. 22: Median plasma βTG concentration and percentage of patients with plasma βTG concentrations > 80 ng/ml in 28 patients with venous thromboembolism for 1 day before and 6 days after commencing heparin therapy.

Both the median βTG concentration and the incidence of abnormal concentrations fell over the first 4 days but rose to a peak again on day 5 of heparin therapy. None of these changes reached statistical significance. These trends are much less evident if one looks at individual patient's results as the concentrations tended to fluctuate quite widely from day to day. Fig. 23 demonstrates this in 5 patients with iliofemoral DVT and PE. Of these 28 patients, 21 show this fluctuating pattern; 4 showed consistently normal concentrations and 3 showed consistently elevated concentrations. There was no correlation between the presence of persistently elevated concentrations and the extent or duration of the thromboembolic process.

Two patients (28 and 32) were treated by thrombolytic therapy. Each day 60 mg plasminogen was given over 4 hours followed by 600,000 units of streptokinase over 30 mins. The βTG concentrations are shown in Fig. 24. In both cases the concentrations rose into the abnormal range after commencing therapy. Therapy for patient 32 was discontinued after 3 days when she developed profuse gastrointestinal haemorrhage and patient 28 showed no change in the extent of the thrombus on repeat phlebography after 1 week of therapy.

B. *Urinary βTG*

The urinary βTG concentrations and excretion rates in all 18 patients in group 1 (controls) are shown in Table 13 and for group 3 (thromboembolism patients) in Table 14.

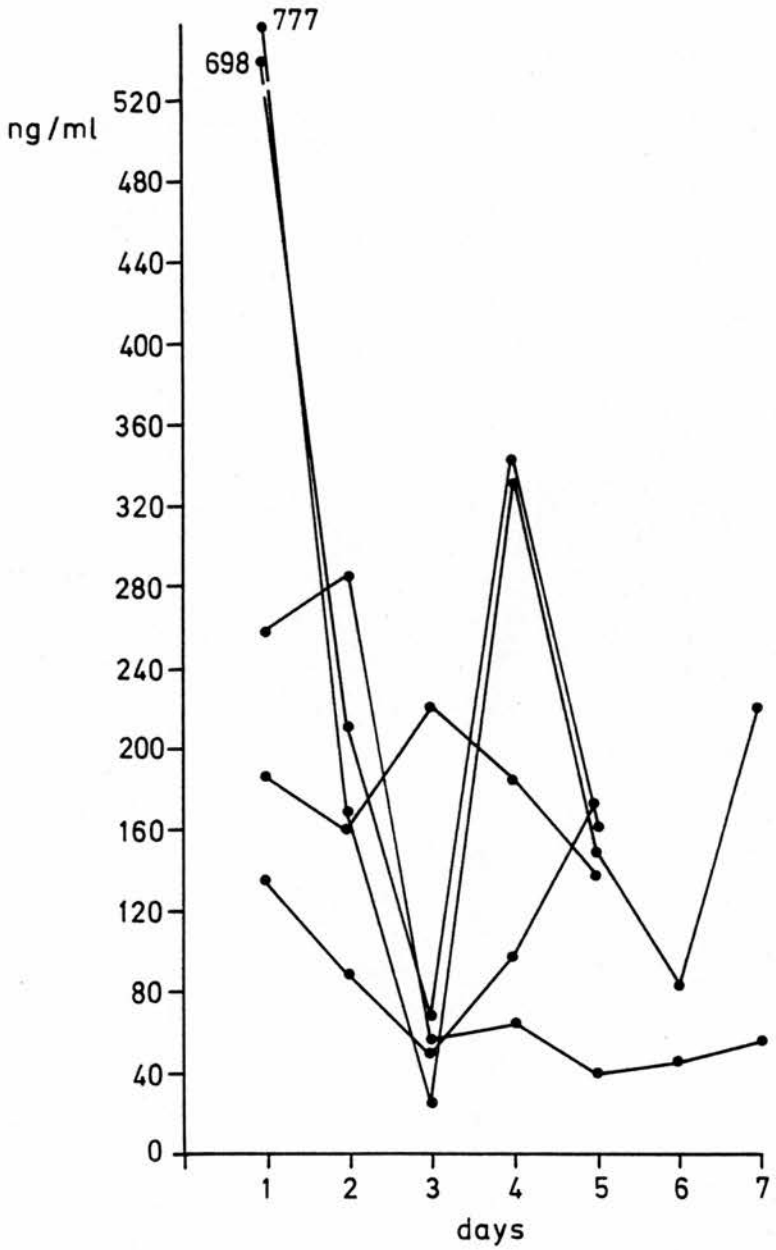

Fig. 23: Plasma βTG concentrations in 5 patients with iliofemoral venous thrombosis and pulmonary embolism.

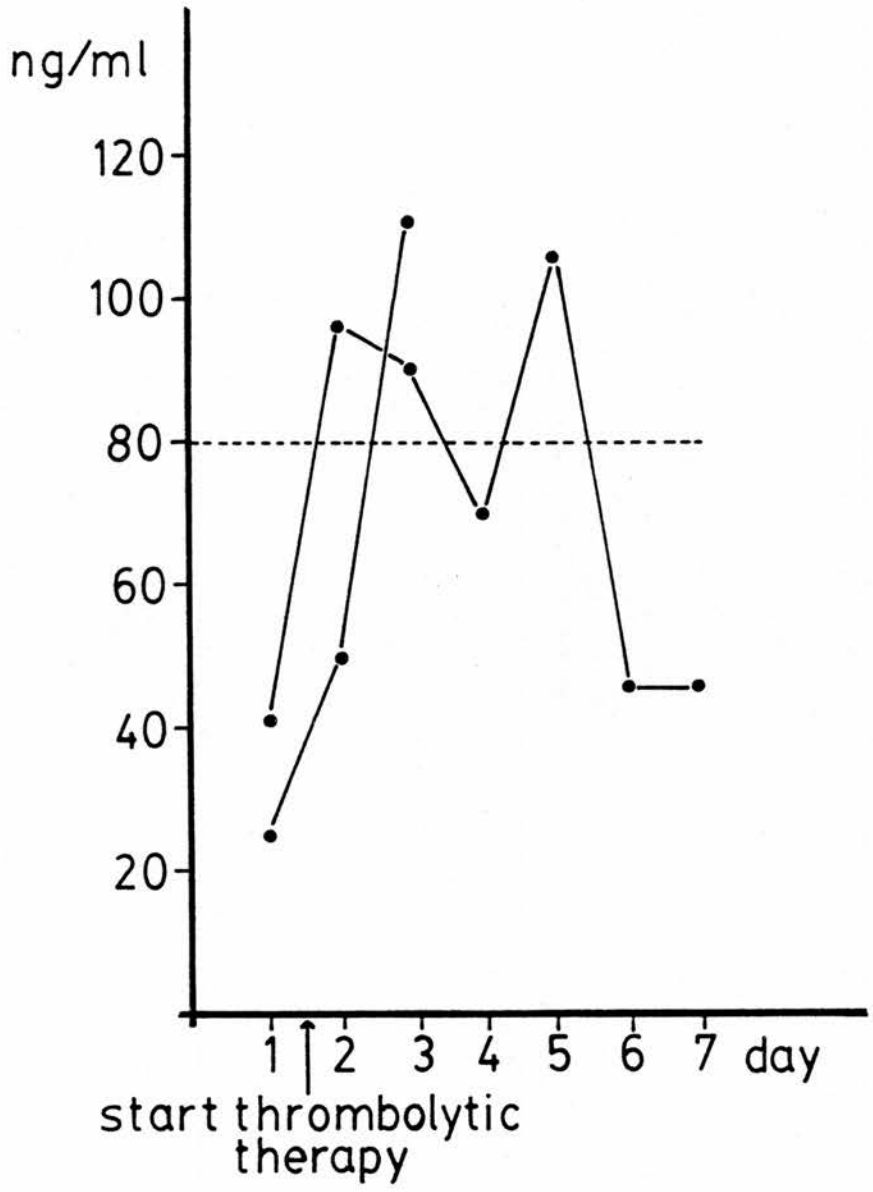

Fig. 24: Plasma βTG concentrations before and after the start of thrombolytic therapy in 2 patients.

TABLE 13: PLASMA AND URINE βTG CONCENTRATIONS AND URINARY βTG EXCRETION RATE IN CONTROL SUBJECTS FOR UP TO 3 DAYS

No.	Plasma βTG		Urine βTG		βTG Excretion Rate	
	Day 1 ng/ml	Range ng/ml	Day 1 ng/ml	Range ng/ml	Day 1 ng/hr	Range ng/hr
1	55	55-100	0.05	0.05	4.3	4.3-6.1
2	245	-	0.10	-	6.2	-
3	(1725)	-	0.08	-	3.5	-
4	76	-	0.12	-	8.0	-
5	(1012)	-	0.08	-	4.7	-
6	110	-	0.11	-	6.2	-
7	45	-	0.10	-	5.3	-
8	24	-	0.15	-	6.4	-
9	114	-	0.13	-	6.0	-
10	22	20-139	0.09	0.05-0.11	4.2	3.0-5.7
11	47	-	1.0	0.32-1.0	47.0	23.0-47.0
12	118	70-118	2.0	0.89-2.0	83.0	31.0-83.0
13	128	-	0.17	0.13-0.17	9.5	7.4-9.5
14	99	-	0.14	0.14-0.69	11.0	11.0-29.5
15	49	-	0.12	0.12-0.15	7.8	7.8-8.2
16	45	45-202	0.73	0.73-1.81	50.2	50.2-79.6
17	222	-	0.69	-	47.1	-
18	56	-	0.65	0.65-0.93	33.9	33.9-64.2

TABLE 14: PLASMA AND URINE βTG CONCENTRATIONS AND URINARY βTG EXCRETION RATE IN THROMBOEMBOLISM PATIENTS FOR UP TO 3 DAYS

	Plasma βTG		Urine βTG		βTG Excretion Rate	
No.	Day 1 ng/ml	Range ng/ml	Day 1 ng/ml	Range ng/ml	Day 1 ng/hr	Range mg/hr
41	90	51-91	0.14	0.13-1.51	14.4	7.5-106.9
45	64	38-576	0.96	0.07-0.96	82.0	1.3-82.0
46	91	37-860	0.31	0.31-2.53	27.3	27.3-120.9
47	16	-	2.5	0.10-2.5	84.0	9.0-84.0
48	338	338-400	0.61	0.60-1.26	20.1	9.6-84.0
49	135	49-135	1.02	1.02-2.3	75.3	75.3-197.6
50	94	50-2686	3.52	0.84-5.32	57.5	57.5-210.0
51	41	-	0.76	0.76-3.7	38.0	38.0-229.4
52	106	-	2.9	2.2-2.9	137.5	115.0-137.5
53	89	78-89	1.5	0.25-1.5	53.8	17.8-152.8
54	4000	-	3.7	1.4-3.7	187.0	74.6-187.0
55	195	117-195	2.5	1.6-2.5	120.6	94.3-120.6
56	55	55-103	0.67	0.35-0.67	87.0	54.4-87.0
57	86	72-86	0.3	0.16-7.9	30.0	5.3-197.5
58	99	-	0.42	0.23-0.42	51.3	29.8-51.3
59	163	-	0.9	-	107.0	-
60	201	-	0.63	0.14-0.63	51.0	8.0-51.0
61	450	310-450	2.6	2.6-5.3	106.4	97.3-183.0
62	69	23-69	1.95	1.36-5.57	163.5	102.0-238.7
63	187	160-300	1.5	1.5-2.0	102.0	82.0-124.0
64	108	94-142	2.37	0.66-2.37	95.0	20.4-95.0

1. *Urine βTG concentration on day of presentation.* The urine βTG concentrations on the day of first presentation are summarised in Fig. 25. The concentrations varied from 0.05 to 2.0 ng/ml in the control group (1) and from 0.14 to 3.7 ng/ml in the thromboembolism group (3). Normal concentrations were found in 72% of the 18 patients in group 1 but in only 5% of the 21 patients in group 3. This difference was highly significant [$\chi^2 = 21.37$, $p = 0.000013$]. The βTG excretion rate varied from 3.5 to 83.0 ng/hr in group 1 and from 14.4 to 187.0 ng/hr in group 3. The difference in incidence of abnormal excretion rates was again highly significant [$\chi^2 = 21.37$, $p = 0.000012$].

There was also a significant difference in the actual concentrations between group 1 (median 0.12 ng/ml) and group 3 (median 1.02 ng/ml) and of the βTG excretion rates (medians 6.2 ng/hr and 82.0 ng/hr respectively) ($p < 0.01$, Wilcoxon). There was no relationship between the extent of the thrombotic process or the duration of symptoms and the βTG concentration or excretion rate.

2. *Follow-up and effect of therapy.* Urine samples were obtained for at least 48 hours in group 3 and it was possible to relate the duration of the elevated urine concentrations to the extent of the thromboembolic process. These data are summarised in Fig. 26. None of the patients with iliofemoral thrombosis had elevated urinary concentrations for < 24 hours and only 22% of those with calf thrombosis had elevated concentrations for > 48 hours. The difference in incidence of an elevated urine concentration and of elevated βTG excretion rates for < 24 hours was statistically significant [$\chi^2 = 10.3$, $p = 0.00035$]. There was no relationship

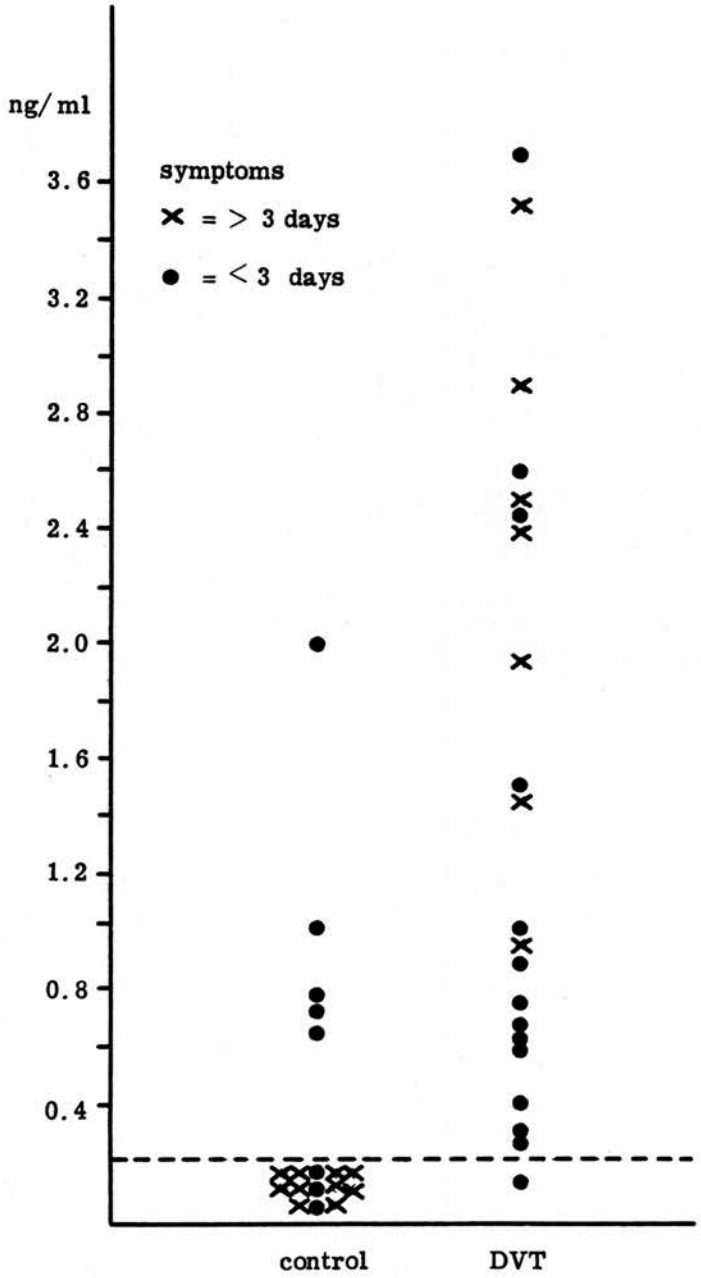

Fig. 25: Urinary βTG concentrations in thromboembolism patients and control subjects on day of presentation.

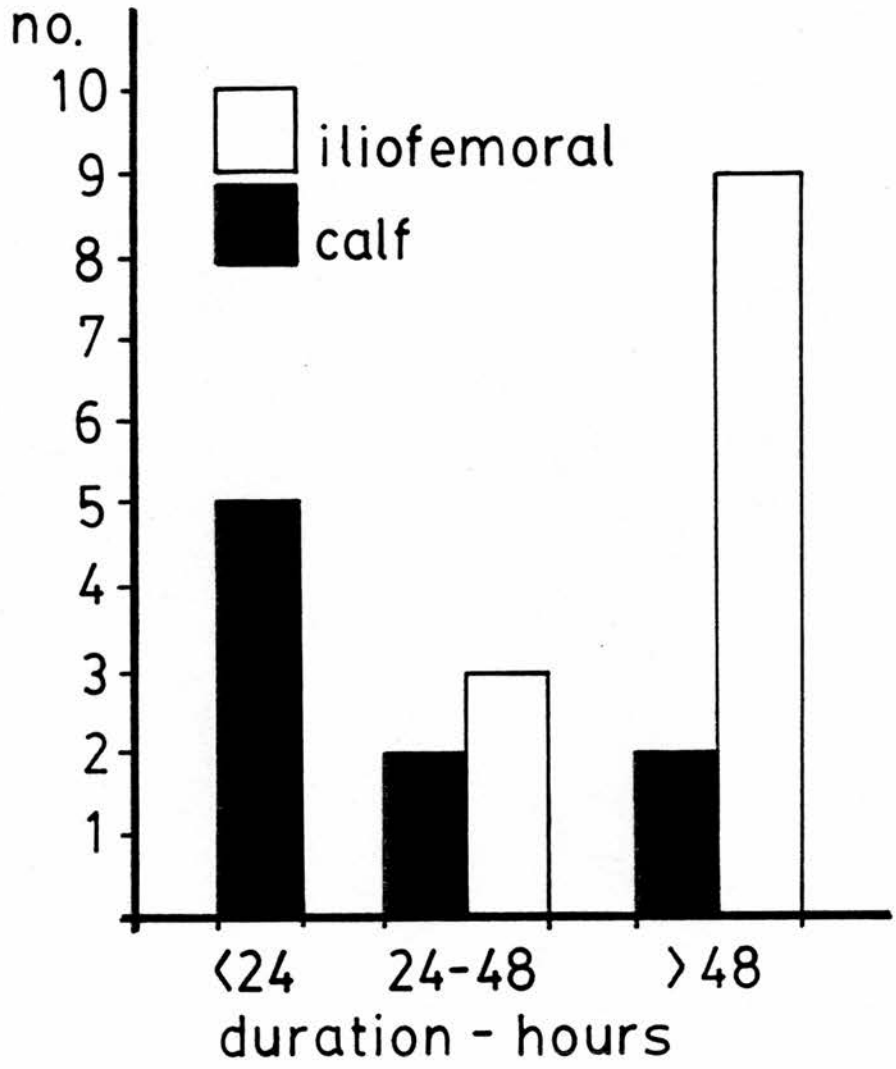

Fig. 26: Duration of elevated urinary concentrations in thromboembolism patients.

between the presence of pulmonary embolism, the aetiology of the DVT or the duration of symptoms and the duration of the elevated urinary concentrations. The one subject who had normal βTG concentrations on admission showed significant elevations within 6 hours (Subject 41, Fig. 27). However, this followed the start of heparin therapy and may be purely a heparin-related phenomenon. The trends of the urinary concentrations tended to vary. In some patients very high concentrations were obtained sometime after initial sampling (patient 59, Fig. 28; patient 40, Fig. 27) whereas in others, particularly those with calf thrombi, the concentration tended to fall rapidly to normal (patient 47, Fig. 27). The βTG excretion rates followed the concentrations quite closely.

Follow-up samples are available in only 9 of the group 1 (control) patients. The 4 patients with abnormal concentrations on presentation (Nos. 11, 12, 16 and 18) still showed elevated concentrations on the following day and one other patient (No. 14) with superficial thrombophlebitis developed elevated concentrations after the first sample.

Seven patients with DVT received no anticoagulant therapy. Six had calf DVT, one associated with pulmonary embolism and one had an iliofemoral DVT. Two of those with calf DVT showed markedly elevated concentrations after initial sampling but in the other 5 subsequent sampling showed lower concentrations. These patterns are demonstrated by patients 59 and 61 (Fig. 28).

The other 14 patients all received heparin therapy. In 7 the therapy had commenced prior to urine sampling and in the other 7 heparin therapy commenced during the period of urine sampling.

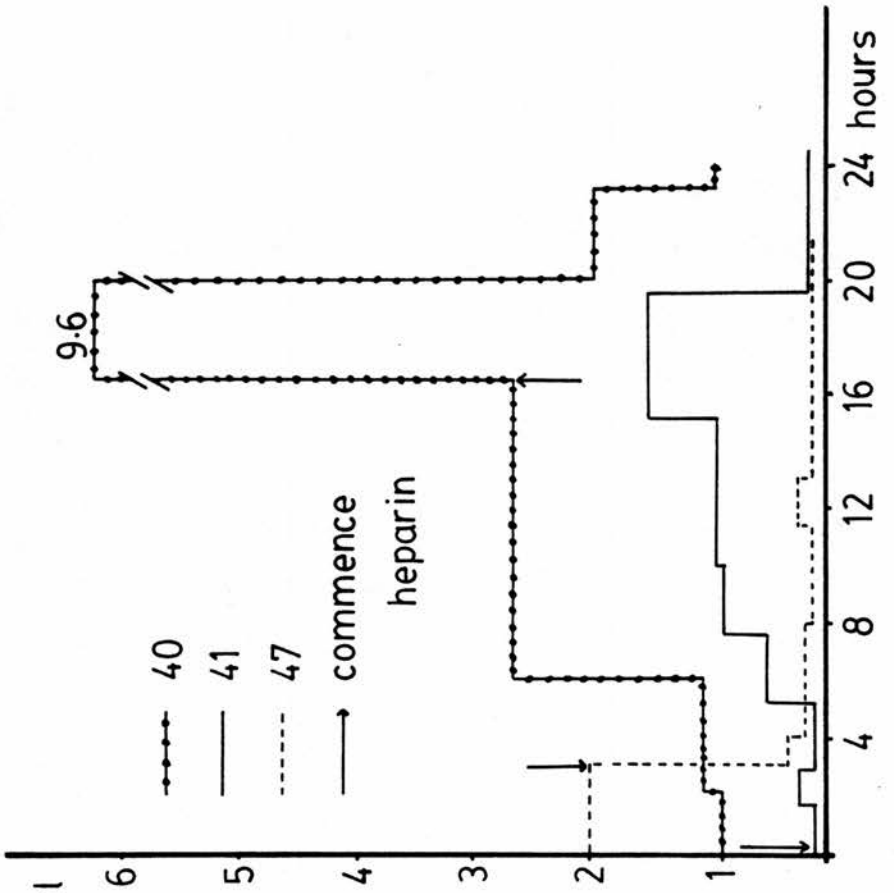

Fig. 27: Urine βTG concentrations in 3 patients (40 iliofemoral DVT, 41 & 47 calf DVT) before and after commencing heparin therapy.

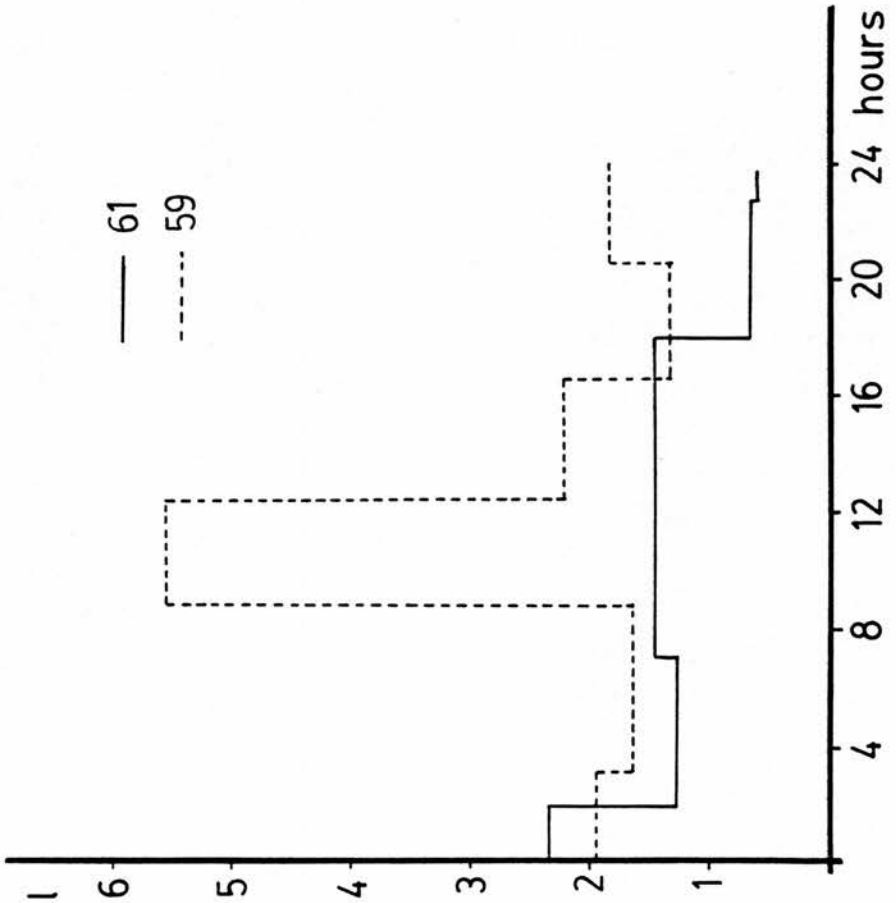

Fig. 28: Urine βTG concentrations in 2 patients (59 calf DVT, 61 iliofemoral DVT) not receiving anticoagulant therapy.

Three patients from each group showed rising concentrations after commencing heparin therapy and 4 showed reductions. Three examples (patients 40, 41 and 47) are shown (Fig. 27). There were 38 plasma samples obtained during urine collections in 18 group 3 patients. There was no correlation between the plasma concentration and the urine concentration ($r = 0.054$) or the βTG excretion rate ($r = 0.091$). The one patient with a normal urine concentration had an elevated plasma concentration and 5 patients with normal plasma concentrations had elevated urine concentrations. There was a similar lack of correlation in 15 samples from 13 patients in group 1 ($r = 0.082$).

Experiment ii: Syringe testing on patients with venous thromboembolism

Syringe tests described in Chapter 1, section 4, measure the rapidity of *in vitro* βTG release. As the release is induced by endogenous thrombin generation the test measures both the rapidity of thrombin generation and the sensitivity of platelets to thrombin.

Methods:

Syringe tests were performed on 3 groups of patients. Their details are summarised in Table 15.

Group 4: consisted of 5 patients aged from 48 to 67 years (mean 58.4 years) presenting with acute DVT. There were 3 women and 2 men, of whom 2 had post-operative iliofemoral thrombosis confirmed by ultrasound; 2 had calf and femoral vein thrombosis

TABLE 15: SYRINGE TEST DATA ON THROMBOEMBOLISM PATIENTS

Group	No.	Age	Sex	Diagnosis	Basal concentration ng/ml	Release time mins
4	65	67	M	Ilio-femoral DVT	188	5
	66	59	M	Ilio-femoral DVT	80	6
	67	48	F	Calf & femoral DVT	64	6
	68	62	F	Ilio-femoral DVT	70	6
	69	56	F	Calf & femoral DVT	83	4
5	70	69	F	2/12 after ilio-femoral DVT & PE	66	4
	71	54	M	5/12 after ilio-femoral DVT & PE	27	6
	72	58	M	4/12 after ilio-femoral DVT & PE	42	3
	73	63	F	2/12 after ilio-femoral DVT & PE	34	8
6	74	48	M	Recurrent DVT & PE	34	5
	75	50	M	Recurrent DVT & PE	24	9
	76	34	M	Recurrent phlebitis	39	7

after surgery diagnosed by ^{125}I fibrinogen uptake test and one had 'spontaneous' femoral vein thrombosis confirmed by phlebography. Sampling was carried out as soon as the diagnosis was suspected clinically.

Group 5: comprised 4 patients, 2 males and 2 females, aged 54 to 69 years (mean 61 years) studied 2.5 months after phlebographically-proven iliofemoral venous thrombosis. Patients 70, 72 and 73 also had pulmonary embolism confirmed by perfusion lung scan. Two patients (70 and 71) had had inconsistently elevated plasma βTG concentrations at the time of the acute episode but the other 2 (72 and 73) had had consistently elevated concentrations. Sampling was carried out at routine follow-up and none of them had had recent acute symptoms. All were anticoagulated with Warfarin.

Group 6: consisted of 3 male patients aged 34 to 50 years (mean 44 years) who had recurrent thromboembolic problems. Patient 76 had had numerous attacks of superficial thrombophlebitis and at the time of sampling was on no medication. The other 2 patients had each had at least 3 episodes of DVT and pulmonary embolism in the previous 2 years and were on Warfarin therapy at the time of sampling. None of the 3 patients had had acute symptoms within the preceding 2 months.

Results:

Table 15 also shows the results of the investigation.

Group 4: (patients with acute DVT). The basal concentrations were high with 3 falling in the abnormal range. The release times

were all rapid. The mean release time was 5.4 mins which was much shorter than our normal range (mean 7 mins).

Group 5: (patients after clinical recovery from acute DVT). The patients in this group all had normal basal concentrations but the release times varied from a very rapid 3 mins to a normal 8 mins (mean 4.8 mins). The release time was not related to the time elapsed since the DVT ($r = 0.23$) nor to the consistency with which elevated plasma βTG concentrations were detected during the acute episode.

Group 6: (patients with recurrent thromboembolic disease). All the basal concentrations and release times were within the normal range.

DISCUSSION

The clinical diagnosis of DVT is notoriously unreliable, especially if the thrombus is confined to the calf when 50% of thrombi may be asymptomatic.[94] It is, therefore, necessary to perform a confirmatory diagnostic test.

Thirty-nine patients had the diagnosis of DVT confirmed by phlebography which is accepted as the most accurate technique available.[171] The accuracy, however, depends on the skill of the radiologist as it has been shown that up to 19% of phlebograms may have some veins incompletely filled concealing minor thrombi.[79,171] We were fortunate in being associated with radiologists particularly experienced in phlebography.

The ^{125}I fibrinogen uptake test, used in 2 patients, has a well established place in the diagnosis of the developing DVT,[137]

but its use is confined to the leg below the inguinal ligament as the background radiation from the pelvic blood vessels masks the counts from the iliac veins. However, in our studies of large numbers of patients with thromboembolic disease, it was extremely rare to find iliac thrombus without co-existing thrombus below the inguinal ligament (< 1% of patients with phlebographically-proven DVT).

Doppler ultrasound is also of value in the diagnosis of DVT particularly in the iliofemoral segment where its accuracy is over 80%.[120] We have found it to be very valuable in patients with occlusive iliofemoral DVT.[124] This test confirmed iliofemoral DVT in 3 patients.

The clinical diagnosis of pulmonary embolism is similarly unreliable and, therefore, chest radiographs and perfusion lung scans using ^{99m}Tc labelled microspheres of human albumin were performed. False negative perfusion scans are exceptionally rare but false positive scans commonly occur with conditions such as pneumonia, atelectasis and pleurisy.[103] However, if one accepts a perfusion defect in the presence of a normal chest radiograph as evidence of pulmonary embolism, the false positive rate is < 10%.[201] The most accurate way of excluding the false positives is to perform a simultaneous ventilation scan which should be normal in pulmonary embolism but show ventilation defects in most of the other pathologies. Unfortunately ventilation scanning was not available. However, I feel confident that the diagnosis of venous thrombo-embolism has been established beyond reasonable doubt in my 46 patients. In the 18 patients in the control group, only 7 patients

were investigaged by phlebography to exclude a clinical diagnosis of DVT. Clinicians were understandably unwilling to subject patients to a rather painful and expensive investigation unless there were definite clinical indications. The patient with superficial thrombophlebitis had small amounts of thrombus in the superficial veins but the deep veins were clear. The other patient with calf pain and a history of two previous episodes of DVT showed poor filling of the deep veins but no obvious thrombus. This illustrates one of the deficiencies of phlebography in that, if there has been a previous thrombosis, it can be difficult to differentiate between old and new occlusive thrombus. The other 11 patients were accepted into the control group on the basis of a definitive diagnosis of a pathology other than venous thromboembolism, as a cause of the symptoms. Therefore, in these 11 patients, DVT as a cause of symptoms has not been completely excluded but is nevertheless extremely unlikely. They also had no clinical evidence of arterial disease.

The comparison of the thromboembolism groups with the control group showed that the 3 groups were matched for age and sex and duration of symptoms.

There was a high incidence of normal plasma βTG concentrations in patients presenting with thromboembolism (35%) in contrast to Ludlam's findings.[107] Other authors have also found disappointingly high incidences of normal concentrations in patients with acute DVT. Denham[42] showed that patients with acute DVT had significantly elevated concentrations compared to controls but failed to record the sensitivity which I regard as an important criterion for judging

the value of a test. O'Brien[147] quoted similar results but his plasma βTG concentrations in DVT (7-240 days after onset) showed complete overlap with his control group. Cella *et al.*[31] produced similar results to the present study and showed that only 11 out of 24 patients with 'recent' DVT had elevated concentrations although the concentrations of the group in general were significantly greater than their control group. They made no comment on how recently their patients had developed DVT. However, the time of development of symptoms may not necessarily be the time of origin of the thrombus. Kakker[94] has shown that up to 50% of legs with evidence of thrombus on ^{125}IFT are asymptomatic and so the development of symptoms may represent a relatively small extension of the thrombus blocking a vital collateral pathway such as the profunda femoris vein. In this situation the majority of the thrombus is old and so the release of βTG will be relatively insignificant. This could well be the reason for the high proportion of false negative results on the samples obtained on the day of presentation. Although many of our patients had recent onset of symptoms we may, in fact, have been studying predominantly old thrombi. Ludlam[107] makes no comment on the duration of symptoms in his patients.

I could detect no significant difference between the βTG concentrations in the patients with calf or iliofemoral thrombosis. Any differences, however, could be marked by the varying age of the thrombus at time of presentation. However, the patients with proven DVT and pulmonary embolism had significantly higher plasma βTG concentrations than those with DVT alone. This is consistent

with the findings of Thomas *et al.*[181] who demonstrated the deposition of platelets on pulmonary emboli and the release of serotonin which was responsible for the bronchoconstriction. They suggested that this aggregation of platelets on the emboli was due to residual thrombin in the embolus and showed that it could be prevented by heparin. Thrombin will also release βTG and, therefore, higher βTG concentrations in the presence of pulmonary emboli should be expected.

The βTG concentrations in the control group were higher than my normal range (Chapter I, Fig. 5). In three of the patients with high concentrations (12, 13 and 14) there was acute pathology which could have been associated with βTG release. Another three (2, 6 and 9) did not have acute processes but were older (aged 48, 61 and 60 years respectively) than the patients in my normal range (mean 29.4 years). Ludlam[109] has shown a tendency to increasing βTG concentrations with age and these values may merely be a manifestation of platelet activity due to mild arterial disease not detected clinically. The seventh was a post-operative patient and is discussed in Chapter III.

During the follow-up period the βTG concentrations fluctuated considerably in most patients possibly indicating episodic platelet activity as new thrombus was laid down. There appeared to be no difference between the patients who received heparin in the first 3 days and those who did not. In the study of normal individuals on heparin therapy, one subject receiving intravenous heparin showed elevated βTG concentrations 2 and 8 hours after injection but the other had only minimal rises. However, the βTG concentration in

the DVT patients on heparin therapy showed no rise immediately after starting heparin therapy. The median concentration fell initially but rose again on day 5 (4 days after commencing heparin therapy). The rise was quite striking but did not reach statistical significance. Although no patients showed overt major bleeding disorders, this secondary rise in βTG concentration could be due to platelet release in relation to sub-clinical internal haemorrhage. Mant *et al.*[116] have shown that bleeding complications are rare in the first 2 days of therapy but that occult retroperitoneal haemorrhage is a frequently unrecognised event in the subsequent days. The effect of heparin on platelet function has already been reviewed in section 5 of Chapter I.

The response of the plasma βTG concentrations to thrombolytic therapy was quite striking but unfortunately only 2 patients were available for study. In 1 patient (No. 32) profuse haemorrhage on day 3 of therapy forced discontinuation of the regime. The rise in βTG concentration in this patient could be merely a manifestation of the developing haemorrhagic diathesis. The other patient, however, showed no evidence of haemorrhage and had no significant changes of haematocrit or platelet count.

Information in the literature on the effect of streptokinase, plasminogen and fibrinogen degradation products (FDP) on platelet function is also rather confusing. Kowalski *et al.*[102] showed that streptokinase and plasminogen or FDP inhibited platelet aggregation and viscous metamorphosis. The early degradation products of fibrinogen appeared to have a more pronounced effect than the later ones. However, they also showed that the platelet count fell after

a dose of streptokinase and plasminogen and demonstrated that this was due to platelet clumping. Kopec *et al.*[101] found that streptokinase and plasminogen inhibited both aggregation and release, as also did FDPs. Morse *et al.*[128] and Millar *et al.*[122] both showed that plasmin inhibited aggregation but not release in human platelets and Martin *et al.*[118] were able to confirm that plasmin had no effect on the release action. In a study with rabbit platelets, Hirsh *et al.*[78] showed that streptokinase infusion had no effect on adhesion or aggregation of platelets. Early FDP, however, inhibited thrombin induced aggregation. The consensus in the literature so far reviewed suggests that thrombolytic agents (streptokinase and plasminogen) and their products (plasmin and FDP) either inhibit or have no effect on platelet function or release. The only data giving evidence to the contrary that I can trace is work by Barnhart *et al.*[8] who showed that low concentrations of FDP, especially segment D, would produce reversible aggregation and release in dog platelets. Kakker,[95] in a review article on thrombolytic therapy comments briefly, without giving experimental details, that low doses of urokinase can increase the tendency to aggregation in animal and human platelets. Although these factors may be responsible for the markedly elevated βTG concentrations detected in subject 28, he did manifest evidence of immunological response to the infused streptokinase with pyrexia and rigors following each dose despite cover with hydrocortisone. This is likely to be associated with circulating immune complexes and these have been shown to induce ADP release from platelets.[129,130] It is possible that βTG could also be released by this mechanism.

An alternative factor could be a diminished vascular integrity similar to that seen with heparin therapy. If vascular integrity is dependent on the fibrin system as suggested by Rowsell *et al.*[167] thrombolytic therapy, especially with the hyperplasminaemia induced by streptokinase, is likely to have an adverse effect and could result in significant βTG release from the platelets escaping from the circulation.

In the 21 patients with DVT in whom urine sampling was performed, only one patient had a normal urinary βTG concentration on the day of presentation. This contrasted with the 13 out of 18 control patients who had normal urinary βTG concentrations. The one patient with DVT who had normal βTG concentration had a localised calf thrombus but subsequent sampling showed significantly elevated concentrations on several occasions. In two patients with DVT the only elevated concentration was obtained with the first specimen on presentation. Therefore, in these two patients I was lucky not to have missed the elevated urinary concentration. Two of the control patients with elevated concentrations on presentation could well have had intravascular thrombosis. Inflammation is associated with a marked vascular response[193] including endothelial cell damage and altered capillary permeability. With particularly virulent infections the inflammatory response may proceed to frank gangrene[66] which will be associated with extensive vascular thrombosis. Immunological complexes may also be involved in altering platelet reactivity and so there are ample reasons for platelet release to be occurring in the patient with cellulitis. This topic will be discussed more extensively in Chapter III. The other patient had

had previous thromboembolic disease and, as I have indicated, the phlebogram did not entirely exclude further thrombosis. One other patient with superficial thrombophlebitis had normal concentrations on admission but several elevated concentrations were detected on subsequent samplings. This patient did have intravascular thrombosis but in the superficial veins, not in the deep ones. Abnormal urinary βTG, like plasma βTG, is unable to differentiate between DVT and other forms of venous thrombosis. Three other patients studied in the post-operative period also had elevated concentrations. This will also be discussed further in Chapter III.

In contrast to plasma βTG assay, the urinary assay was unable to identify those with pulmonary embolism but the patients with ilio-femoral DVT had elevated concentrations and excretion rates for significantly longer than those in whom the DVT was confined to the calf. This probably represents a continuing slow turnover of platelets in the more extensive thrombus leading to a continuous hyperexcretion of βTG over a longer period. The plasma βTG assay differentiated between those with and without pulmonary embolism on the basis of βTG concentration at presentation. This represents greater activity, but in a shorter period. As the proportion of plasma βTG actually excreted in the urine is minute, the difference in plasma concentrations in those with and without pulmonary embolism is insufficient to produce a significant difference in urinary concentrations.

Heparin therapy did not appear to alter the widely differing patterns of urinary βTG concentrations seen in non-anticoagulated patients with DVT. However, none of the patients in the urinary

βTG study were followed for more than four days after presentation and none of the patients reached the point at which the plasma βTG concentrations peaked (five days after commencing therapy).

There appears to be no correlation between the plasma and urine βTG concentrations. In the infusion study it has been shown how good the correlation between plasma and urinary βTG concentrations was but in that study frequent urine and plasma samples were obtained. In the study on the DVT patients the plasma was sampled at some stage during the period the urine was collecting in the bladder, which could be as long as 6 hours. During such a collection in a patient with DVT there may well be considerable variations in the plasma βTG concentration which will be mirrored by variations in the amount of βTG passing into the bladder. As the urine is mixed in the bladder, the ultimate βTG concentration will reflect the 'mean' urinary βTG concentration over the period the urine has been accumulating. The plasma βTG, in contrast, measures the βTG content of the plasma at the moment when sampling is performed. I have shown how the plasma βTG may vary greatly from day to day in patients with DVT. If one sampled during a period of low βTG concentration, one could obtain a false negative result. Urine sampling, however, helps to eliminate these spurious negative results by compensating for periods of low excretion with periods of high excretion.

Despite the disappointing results with the syringe tests in normals, the studies in those with thromboembolism did seem to confirm the findings of others. The patients sampled during the acute phase of a DVT showed high basal concentrations and

significantly shorter release times ($p < 0.05$, Wilcoxon) than the normal group. This suggests that the platelets are more reactive during the acute phase of DVT. Abrahamson,[2] Harker[74] and O'Brien[146] have all shown that platelet survival is decreased during the acute phase of DVT. Therefore, the mean age of the circulating platelets will be shorter. Minter and Ingram[125] have shown that, following acute blood loss in the dog, the mean platelet size increases temporarily and they suggest that the newly formed platelets are larger. Karpatkin[97,98] supports this theory and shows that young platelets are more active in that they aggregate faster in response to thrombin and they secrete larger amounts of ADP and PF_4. Manucci and Sharp[117] even believe that only the young large platelets will aggregate. The shortened release time may, therefore, be due purely to a younger platelet population as a result of increased platelet production following platelet consumption by the thrombus.

The release times in the patients convalescent one to four months after acute DVT varied considerably but two of the patients had shorter than normal release times. The release times did not appear to be related to the extent of βTG release during the acute episode or to the time since the acute episode. The ultra short release times (3 and 4 mins) seen in two patients may be significant but the numbers studied are too small to show a trend. Abrahamson,[2] however, showed that platelet survival returned to normal after clinical recovery in patients with DVT.

The release times in the three patients with recurrent thrombo-embolic problems were all within the normal range. This is probably

to be expected as the majority of investigators have incriminated abnormalities of the fibrin generation or fibrinolytic systems rather than the platelet system in this condition. Egeberg[51] demonstrated an inherited deficiency of anti-thrombin and Isaccson and Nilsson[85] showed defective fibrinolysis in blood and vein wall in these patients. The success of anabolic steroids in the prevention of relapses in recurrent superficial thrombophlebitis[87] and their known ability to stimulate release of plasminogen activator from vessel wall suggest that abnormalities of the fibrin generation or fibrinolytic systems rather than platelets are involved. However, Hirsch and McBride[77] found an increased platelet adhesiveness and Steele *et al.*[178] also demonstrated reduced platelet survival in patients with recurrent thromboembolic disease. These changes, however, may be insufficient to produce any change in the syringe tests.

This chapter has been mainly devoted to a study of the value of βTG assays in the diagnosis of DVT and PE. How good are they? If a patient presents with symptoms suggestive of DVT and/or PE, what chance is there that the correct diagnosis will be reached using the βTG assay alone? With one plasma concentration one would have a 65% chance of reaching the correct diagnosis (probably a similar chance to clinical examination) but one urine concentration would correctly diagnose 85% of the patients. The plasma βTG concentration would give a false positive diagnosis in 44% of patients without DVT but the urine βTG assay would diagnose only 28% false positive.

Other assessments for comparative purposes are the sensitivity, specificity, Youden's Index J^{207} and the Δ value.[5] The Youden's Index is a method of aggregating the estimates of the sensitivity and specificity of the tests. It is calculated by subtracing the sum of the false negative and false positive proportions from 1. Therefore, the higher the value the greater the discriminant accuracy of the test. The Δ value is calculated by dividing the difference of the means of the two samples by the square root of the variance of the total population of the βTG concentrations. The results are shown in Table 16. This confirms that in all parameters measured, the urine assay is a better discriminant than the plasma assay. It is also much more attractive because of its simplicity of sampling but is seriously affected by haematuria, proteinuria or operation. This will be discussed further in Chapter III.

TABLE 16: DIAGNOSTIC ACCURACY OF PLASMA AND URINE βTG CONCENTRATIONS IN VENOUS THROMBOEMBOLISM

	Group	Test Result +	Test Result -	Total	Sensitivity	Specificity	True positive + true negative	J	Δ
Plasma	DVT	15	6	21	0.71	0.56	0.65	0.27	0.48
	Control	7	9	16					
Urine	DVT	20	1	21	0.95	0.72	0.85	0.67	0.89
	Control	5	13	18					

CHAPTER III

STUDIES IN SURGICAL PATIENTS

The reliable diagnosis of pathological intravascular thrombosis by a simple blood test would be of considerable benefit in patients undergoing major surgery.

DVT is a common sequel to operation, the incidence varying from 15% in patients undergoing gynaecological procedures[20] to 35% in general surgical patients[62] and 75% in patients with pertrochanteric fractures.[60] PE follows operation in about a quarter of patients[23] but is usually minor and asymptomatic. It may, however, on occasions, be massive and fatal. Patients with evidence of DVT, on ^{125}IFT have almost a one in two chance of developing PE.[23] Early diagnosis of these at risk patients may enable early treatment to prevent this potentially fatal complication.

In patients undergoing vascular reconstruction, assessment of patency of the reconstruction may be difficult if distal occlusions prevent the reappearance of distal pulses. A test for intravascular thrombosis might be of value in this situation to detect occlusion or impending occlusion of a reconstruction, thus enabling appropriate early remedial action. The aims of this study are, therefore, twofold:-

1. To assess the value of plasma and urine βTG measurements in the diagnosis of post-operative DVT.
2. To assess the value of plasma βTG measurements in the diagnosis of post-operative thrombosis of arterial reconstructions.

However, before assessing the βTG concentration in these pathological situations, it is important to evaluate the effects of the operation and the many factors which may influence platelet function in the peri-operative period.

The study is divided into six parts:-

1. Pre-operative plasma βTG
2. The effects of operation on plasma βTG
3. The effects of post-operative factors on plasma βTG
4. The diagnosis of post-operative DVT
5. The diagnosis of post-operative thrombosis of arterial reconstructions
6. Peri-operative urinary βTG

1. PLASMA BETA-THROMBOGLOBULIN BEFORE OPERATION

Aim:

To study the plasma βTG concentration in various pathological states before operation and to see if there was any change after admission to hospital.

Methods:

The study group comprised 117 patients admitted to a general surgical unit for major surgery.

Plasma samples were obtained on admission on 116 patients and a second sample was obtained on the following day in 73 of them.

Results:

The patients were divided into two groups. Group GS consisted of 68 patients undergoing general surgical procedures and group PVD comprised 49 patients with peripheral arterial disease. The population statistics are summarised in Table 17. There was no difference in age between the two groups but there was a significant difference in sex distribution, with a male predominance (74%) in the peripheral vascular disease patients and a female predominance (63%) in the general surgical patients [$\chi^2 = 15.4$, $p = 0.00009$].

The distribution of the plasma βTG concentrations on days 1 and 2 before operation and the median concentrations in the various groups are shown in Fig. 29. Day 1 is the day of admission and day 2 the following day. On day 1, 27% of GS group and 33% of PVD group had concentrations in the abnormal range but by day 2 this had fallen to 13% of GS group and 19% of PVD group. There was no

TABLE 17: POPULATION STATISTICS ON SURGICAL PATIENTS

Group	Total	Male	Female	Age range	Mean
GS	68	25	43	22-87	60.3
PVD	49	36	13	42-82	60.7
Total	117	61	56	22-87	60.4

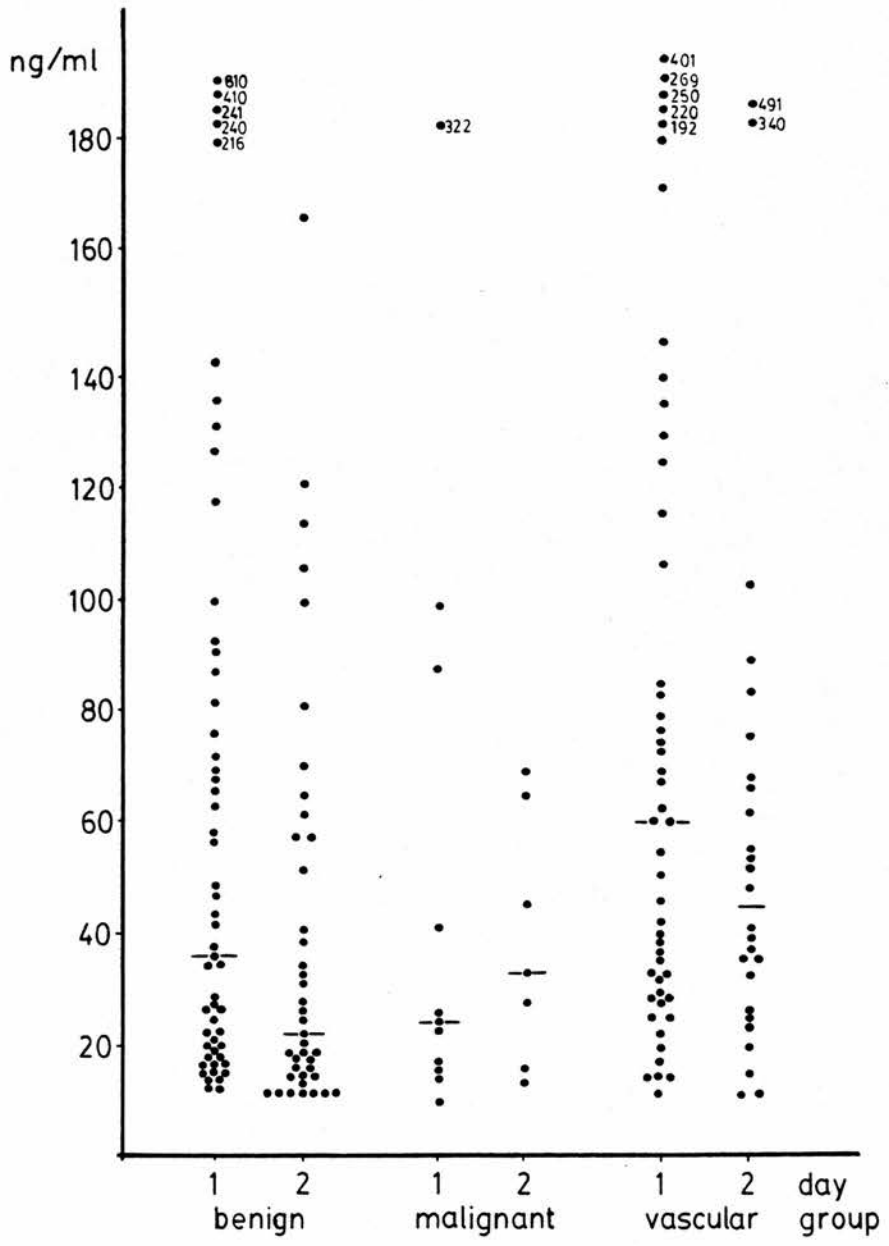

Fig. 29: Plasma βTG concentrations before operation (median concentrations indicated) on days 1 and 2.

difference between the two groups of patients. The subgroup of general surgical patients with malignant disease tended to have lower concentrations than the others. The incidence of abnormal concentrations suggests some temporary effect promoting βTG release on admission to hospital.

In 47 GS group patients, plasma concentrations were available on both days and, of these, 60% showed no elevated concentrations; 36% had one elevated concentration but only 4% had both concentrations elevated. From day 1 to day 2 75% of the patients showed a fall and 25% showed a rise. The correlation between the two days was poor ($r = 0.16$). Similar results were obtained in the 26 PVD group patients sampled on both days: 61% had no elevated concentrations; 31% had one elevated concentration and only 8% had both concentrations elevated. Between days 1 and 2 50% showed a fall and 50% showed a rise. The correlation between the two days in the vascular patients was much better ($r = 0.60$).

Statistical analysis has been performed on these results using a t test on the logarithm of the concentration to normalise the distribution.

GS patients had concentrations on day 1 (median 35 ng/ml, range 10-810 ng/ml) significantly higher ($t = 2.01$, $p = 0.047$) than those on day 2 (median 21 ng/ml, range 12-169 ng/ml) and than the normal range ($t = 4.21$, $p = 4.2 \times 10^{-5}$). The concentrations on day 2 were not different from the normal range ($t = 1.32$, $p = 0.19$). The concentrations on day 1 in the PVD patients (median 60 ng/ml, range 12-401 ng/ml), however, were not different ($t = 0.85$, $p = 0.40$)

from the concentrations on day 2 (median 45 ng/ml, range 12-491 ng/ml), nor were they different from day 1 of GS patients ($t = 1.85$, $p = 0.067$). They were significantly higher than the normal group ($t = 7.09$, $p = 2.3 \times 10^{-10}$) and from day 2 of the GS patients ($t = 4.09$, $p = 8.9 \times 10^{-5}$). On day 2 the concentrations of PVD patients were significantly higher than the normal group ($t = 4.66$, $p = 8.5 \times 10^{-6}$) and day 2 of the GS patients ($t = 2.5$, $p = 0.015$).

Discussion

There was a considerable variation in βTG concentration detected in patients on admission to hospital with about a third of patients having abnormally high concentrations. By the following day only a sixth of the patients still had abnormal concentrations. The peripheral vascular patients showed a greater tendency to abnormal concentrations than the general surgical patients and the concentrations did tend to remain elevated on the second day.

Several factors may be involved in these findings. The patients studied here were much older than the normal group and the rise in βTG concentration may be a manifestation of age as shown by Ludlam.[109] The cause of this may be the development of the multiple endothelial lesions associated with aging arteries. This is borne out by the close correlation between the βTG concentrations in these patients and those with peripheral arterial disease in Chapter IV and by the greater tendency for the PVD patients to have elevated concentrations on both days.

All these patients, unlike the normal group, had some form of continuing pathological process which could contribute to βTG release.

However, this is not compatible with the fall in βTG concentrations seen in the GS patients between days 1 and 2. In these patients some influence seems to be more active immediately after admission than on the following day. Haft and Arkel,[71] in a study on junior doctors presenting papers at academic meetings, have demonstrated that the emotional stress of that situation can affect platelet function. By studying thrombin induced platelet aggregation before and after the presentation of the papers, they demonstrated that platelets aggregate during stress, de-aggregate and are then refractory to re-aggregation.

Admission to hospital is psychologically stressful and this may be sufficient to produce βTG release. By the following day the patient has adapted to his surroundings and should be more at ease thus resulting in a fall in the βTG concentrations on day 2, as shown in the general surgical patients.

2. PLASMA BETATHROMBOGLOBULIN DURING OPERATION

Aim:

To study the effect of operation on plasma βTG.

Methods:

Thirty-nine patients undergoing abdominal or vascular operations were studied. All patients were screened for DVT by the ^{125}IFT. A careful record was kept of all transfusions of blood or blood products. Samples of blood were obtained for βTG assay and platelet count prior to operation, after induction of anaesthesia, on completion of the preliminary dissection, within 10 mins of clamp release in arterial operations or just prior to abdominal closure in general surgical operations, in the recovery room within 30 mins of completing the operation, and daily thereafter.

Results:

The 39 patients were divided into three groups.

Group I: comprised 15 patients (12 males and 3 females) aged 49-75 years (mean 60 years) undergoing major arterial reconstructions such as major endarterectomies or bypass grafts. Of these, 10 received blood transfusions during surgery and 6 developed post-operative DVT, as diagnosed by the ^{125}IFT.

Group 2: comprised 14 patients (11 males and 3 females) aged 42-70 years (mean 60 years) undergoing carotid endarterectomy. No patients received blood transfusions or developed ^{125}IFT diagnosed DVT.

Group 3: consisted of 10 patients (5 males and 5 females) aged 46-81 years (mean 62 years) undergoing major abdominal surgery such as cholecystectomy or bowel resection. Two received a blood transfusion and one developed ^{125}IFT diagnosed DVT.

The median βTG concentrations for each group are plotted in Fig. 30 and the proportions of patients with abnormal concentrations shown in Fig. 31. Only 4 patients (11%) had elevated concentrations before operation.

i) *Effect of induction of anaesthesia.* In 13 patients βTG concentrations were available before and after induction of anaesthesia. There was a small and statistically insignificant rise, with only 3 patients (1 from each group) showing rises of > 30 ng/ml.

ii) *Effect of operation.* The plasma βTG concentrations during operation varied enormously (15-2,283 ng/ml) between individuals. However, the concentrations on repeated sampling in individual patients tended to follow a consistent pattern of release or lack of release.

In those undergoing major arterial operations (group 1) there was a significant rise in βTG concentration relative to the pre-operation levels detected in the first intra-operative specimen ($p < 0.01$), continued through the second intra-operative specimen, and at a lower level in the immediate post-operative specimen. By day 1 after operation the plasma βTG concentration was still slightly elevated but not significantly so. In groups 2 and 3 significant elevations relative to the pre-operative concentrations were found

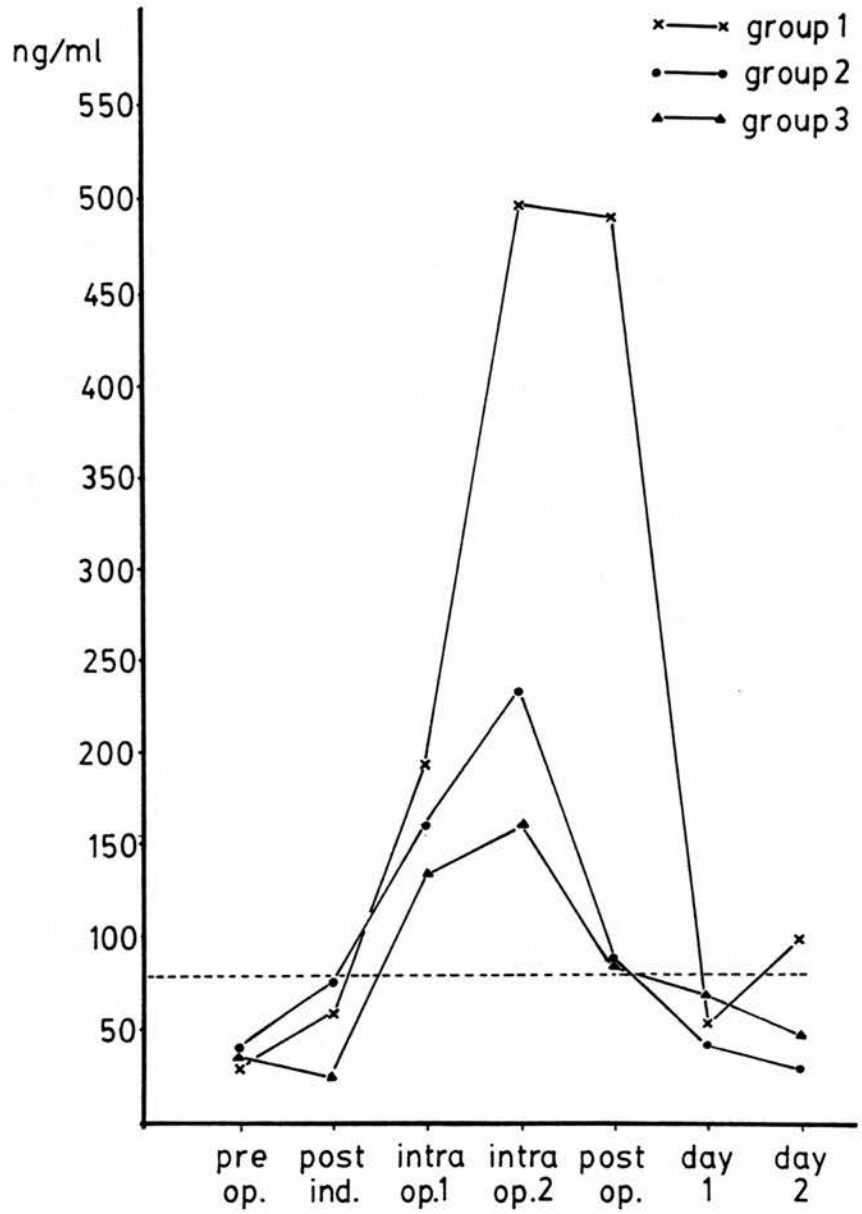

Fig. 30: Median plasma βTG concentrations during operation.

Group 1 - major arterial reconstruction
Group 2 - carotid endarterectomy
Group 3 - major abdominal operations

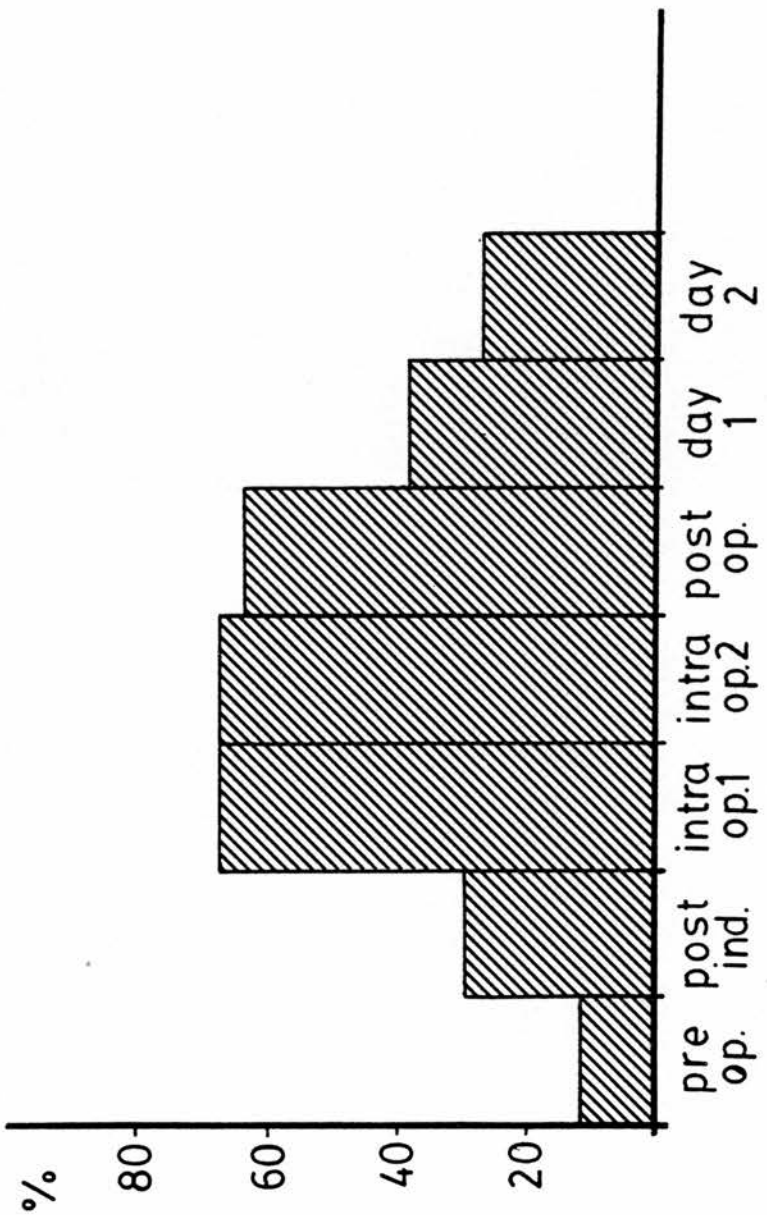

Fig. 31: Proportions of patients with plasma βTG concentrations > 80 ng/ml during operation.

in the intra-operative specimens only ($p < 0.01$).

In comparing the three groups, the patients in group 1 showed higher concentrations than those in groups 2 and 3 but this only reached significance in the immediate post-operation specimens relative to group 2 ($p < 0.01$) and group 3 ($p < 0.05$). In 9 patients (2 in group 1, 5 in group 2 and 2 in group 3) the βTG concentrations never exceeded 80 ng/ml.

The proportions of abnormal concentrations at each point are shown in Fig. 31. These data mirror the medians in Fig. 30.

iii) *Effect of a vascular procedure*. The median βTG concentration in group 1 rose by over 300 ng/ml after the circulation was re-established through a graft or endarterectomised segment. This change failed to reach statistical significance because of the small numbers involved.

iv) *Effect of blood transfusion*. Plasma samples were obtained during the infusion of whole blood or packed cells in 12 patients. There was no difference in concentration between those samples and samples obtained at the same point during the operation in patients not receiving a transfusion.

Discussion

There was again a considerable variation in the βTG concentrations detected in all three groups: major arterial, carotid endarterectomy, general surgical. In 23% of the patients no abnormal concentrations were detected at any stage but all except two of these patients did show rises which failed to reach 80 ng/ml.

These patients all had pre-operative concentrations below 40 ng/ml and it may be that with low initial concentrations the release engendered by surgery was insufficient to raise the level above the normal range. This phenomenon was commoner in patients undergoing carotid surgery (a much less traumatic procedure than a laparotomy or major arterial reconstruction) and is, therefore, related to the extent of the surgical procedure. However, two patients undergoing major arterial surgery (aorto-iliac endarterectomy and prosthetic graft replacement of a femoral aneurysm) also failed to show significant release. A syringe test (Chapter I, section 4) was carried out several months after operation on the patient who underwent aorto-iliac endarterectomy and this showed a release pattern at the upper limit of type I (a very slow release).

The failure to release in some patients may be due to a combination of less traumatic surgery and platelets which are relatively insensitive to releasing effects.

There was a small but statistically insignificant rise in βTG concentration following the induction of anaesthesia and this can be attributed to platelet activity associated with the insertion of venous and arterial catheters and the intravenous injection of irritant anaesthetic agents. Venous thrombosis is a well recognised phenomenon after injection of sodium pentothal, the induction agent used in all the study patients. Gaseous agents such as halothene, diethyl ether and cyclopropane tend to inhibit aggregation of platelets,[185] but the effect appears to be outweighed by the other factors promoting release.

Further release continued as the operations progressed and the concentrations peaked in the second intra-operative specimen. This was the point at which the trauma had reached its maximum. In the vascular surgical patients, the graft or endarterectomised segment had been perfused for 5-10 mins by this stage and this is reflected by the considerable rise in concentration in group 1 between the first and second intra-operative specimens. Evans and Mustard[56] have shown how prosthetic surfaces will induce platelet aggregation and it is likely that there will be extensive aggregation on the surfaces of large prosthetic arterial grafts. Similar activity will also occur on the areas of collagen exposed during endarterectomy as collagen is known to be a potent stimulator of aggregation.[83,84]

In group 1, patients showed significantly elevated concentrations, continuing in the immediate post-operative specimen. It is unlikely to be due to the effect of heparin (as described in Chapter I) as the same dose of heparin was given to group 2 patients who did not demonstrate this phenomenon. More patients in group 1 than in the other groups received blood transfusions but it was not possible to demonstrate any difference between those receiving blood and those not transfused in any of the groups. This, too, is unlikely to explain the persistently elevated concentrations. Another possible factor is the extent of the trauma of surgery but group 3 had a similar extent of trauma to group 1. Therefore, this persistently elevated concentration is most probably due to persisting platelet activity in the graft or endarterectomised segment. Both types of reconstruction had similar release patterns.

Although the group 2 patients had similar endarterectomised segments, the surface area was so small as to be unlikely to produce such a marked effect as in the group 1 patients.

3. PLASMA BETATHROMBOGLOBULIN CONCENTRATION IN THE POST-OPERATIVE PERIOD

Aim:

To study the effects of various factors on plasma βTG after operation.

Methods:

The 117 patients in part 1 of this chapter were also studied in the post-operative period. All patients were screened for DVT by the ^{125}IFT using the technique described in Appendix A. Blood samples were drawn daily for βTG assay and full blood count, including platelet count, for 1-3 days before operation and for up to 10 days afterwards. A careful record was kept of all drug therapy, transfusions, infective complications and the presence of infusion phlebitis. Also recorded were the temperature, pulse rate and results of any investigations of thromboembolic phenomena.

Results:

The two groups, summarised in Table 18, were analysed separately.

i) *Population statistics*

a) General surgical patients (GS). Six patients in this group were technically unsatisfactory leaving 62 patients of whom 48 (29 female and 19 male) had negative ^{125}IFTs and 14 (10 female and 4 male) had positive ^{125}IFTs.

The operations performed on these patients are summarised in Table 18. The incidence of DVT in patients with malignant disease (70%) was higher than in those with benign disease (13%).

TABLE 18: GENERAL SURGICAL PROCEDURES AND DVT INCIDENCE

Surgical procedure	Positive $^{125}_{IFT}$	Negative $^{125}_{IFT}$
a) *Benign disease*		
Cholecystectomy ± duct exploration	3	27
Gastric procedures	2	13
Division of adhesions	2	1
Colon resections	-	4
b) *Malignant disease*		
Gastroenterostomy	1	-
Bilateral adrenalectomy	1	-
Colon resections	2	2
Exploratory laparotomy ± colostomy	3	1
Total	14	48

TABLE 19: VASCULAR OPERATIONS

Operation	No.
Carotid endarterectomy	20
Aorto-iliofemoral Dacron graft	11
Aorto-iliac endarterectomy	6
Peripheral minor Dacron grafts	6
Femoro-popliteal vein bypass	6
Total	49

b) Arterial surgical patients (PVD). Six patients in this group who underwent femoro-popliteal bypass were excluded as it was not possible to scan the operated leg and a further four patients who developed major post-operative complications have been analysed separately. This left 39 patients of whom 28 (21 male and 7 female) had negative ^{125}IFTs and 11 (6 male and 5 female) had positive ^{125}IFTs. The operations performed on these patients are summarised in Table 19.

ii) *Patients with uncomplicated post-operative progress*

Sixteen GS patients and 9 PVD patients had no post-operative complications and received no heparin in the post-operative period. Their post-operative βTG concentrations are shown in Figs. 32 and 33. The post-operative follow-up in the PVD patients is short as the majority of the carotid endarterectomies, who form the bulk of this group, were discharged within 4 or 5 days of operation.

In the GS patients the βTG concentration shows an early fall with a later rise to peak on day 6. The rise did not reach statistical significance. The PVD patients showed a similar pattern but peaked on day 4. If the effect of the post-operative rise in platelet count is eliminated by plotting the results as the βTG/platelet ratio, a similar pattern is obtained (Figs. 34 and 35). This suggests that the rise cannot be attributed to an elevated platelet count alone.

iii) *Infusion phlebitis*

Thirteen GS patients developed tenderness and/or erythema over a vein in which an intravenous infusion was given. The plasma βTG

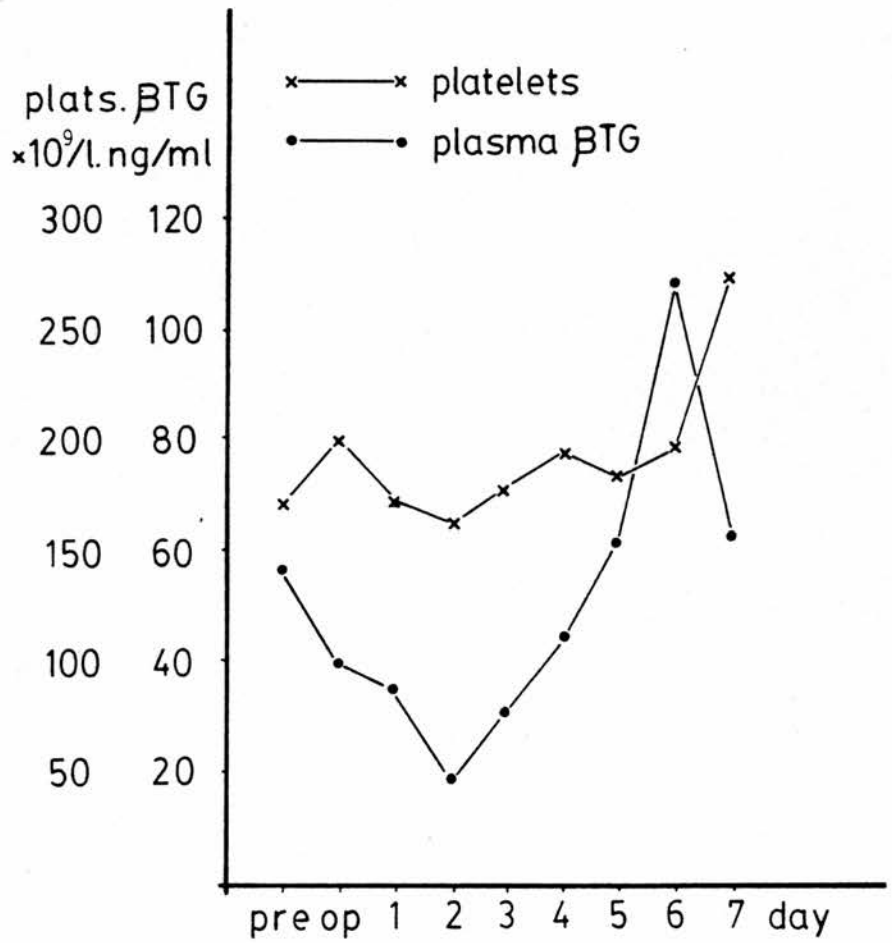

Fig. 32: Median plasma βTG concentrations and platelet counts in patients without complications after general surgical operations.

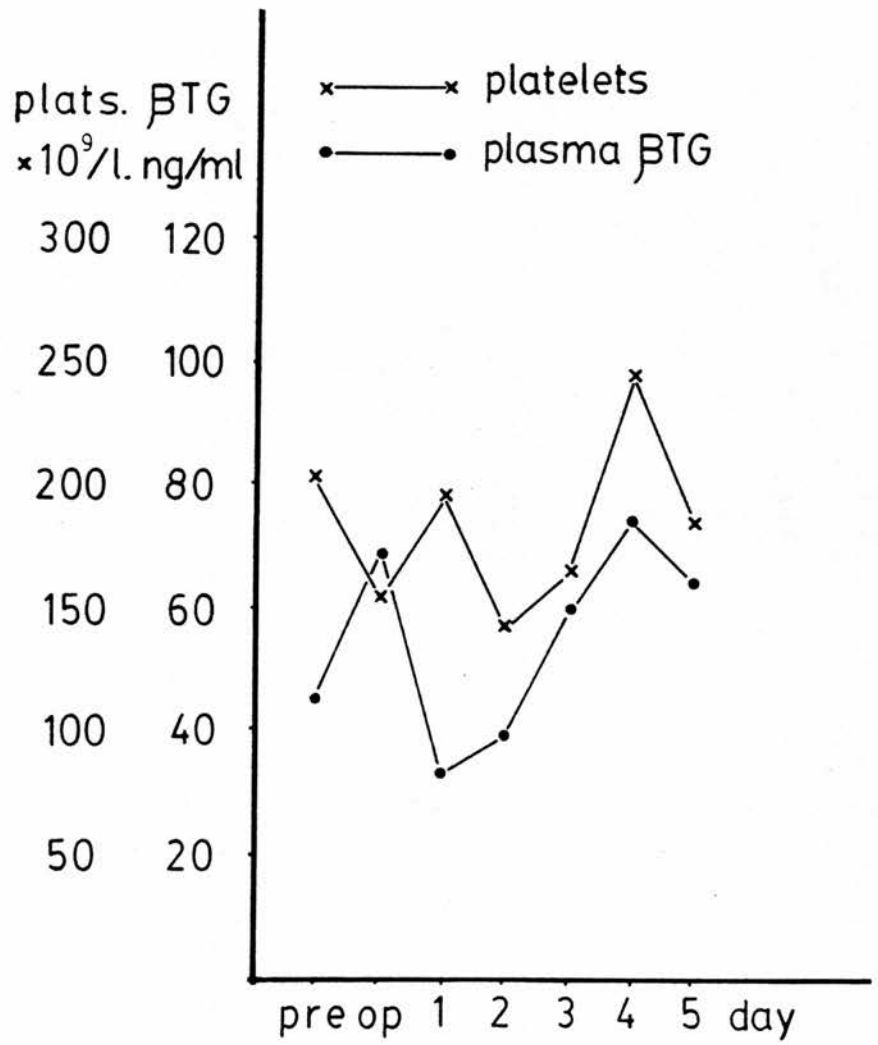

Fig. 33: Median plasma βTG concentrations and platelet counts in patients without complications after arterial operations.

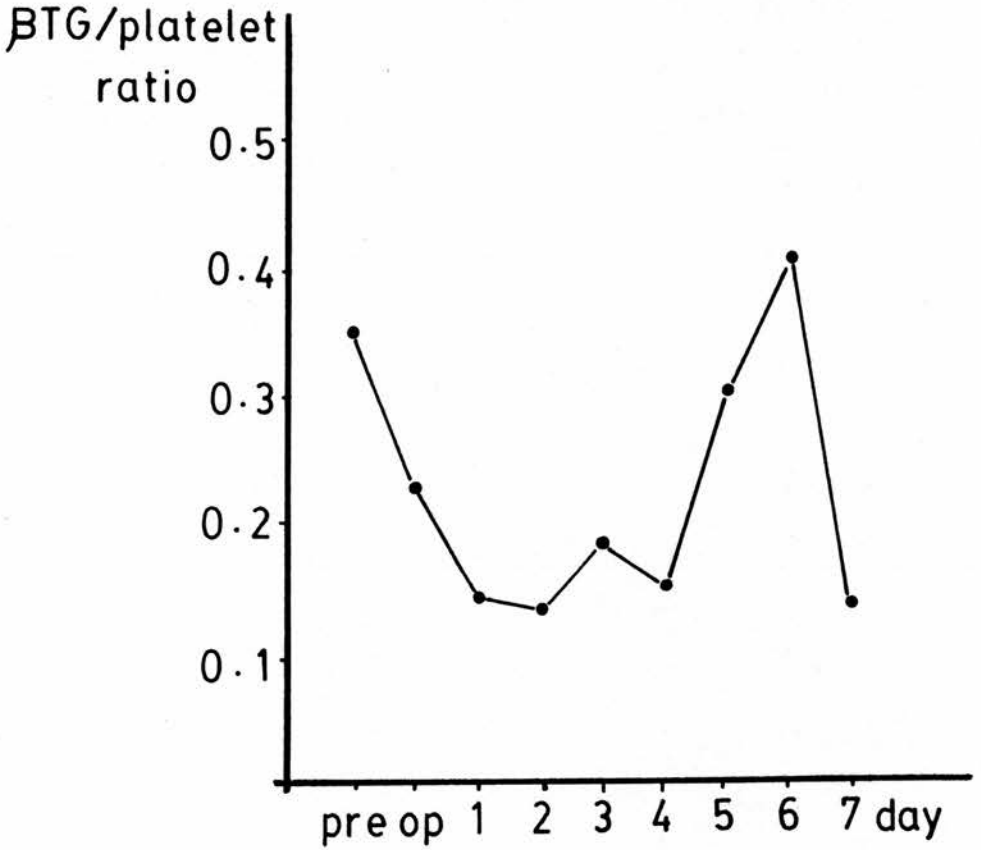

Fig. 34: Median plasma βTG/platelet ratio in patients without complications after general surgical operations.

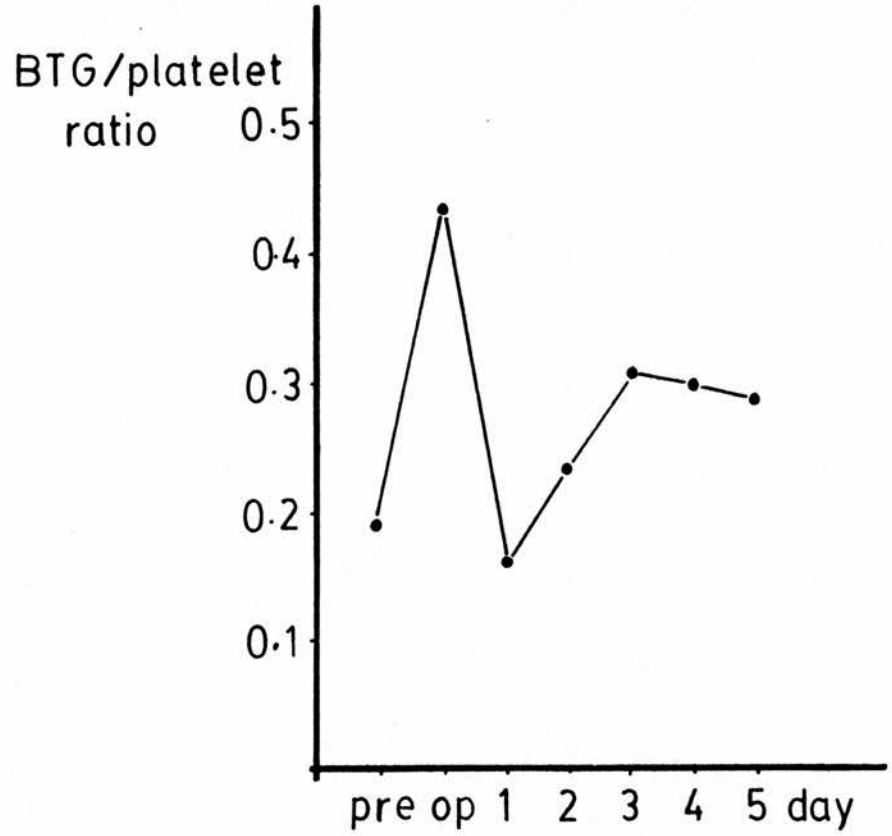

Fig. 35: Median plasma BTG/platelet ratio in patients without complications after arterial operations.

concentration showed a small but statistically insignificant rise 2 days after the phlebitis became clinically apparent.

iv) *Drugs and blood transfusion*

Numerous drugs were given in the post-operative period and no consistent relationship with βTG release could be identified. Only two patients received blood in the post-operative period. Both showed slightly elevated concentrations (80 and 159 ng/ml) during the transfusion.

v) *Infection*

Seven patients developed clinically significant infections. In two of these the infection was minor and localised. One had a cough with purulent sputum and pyrexia of 38^0C but no changes were detectable on chest radiograph. The other developed a localised wound abscess which discharged spontaneously. The other five patients developed serious infections: three had lobar pneumonia with pulmonary consolidation on radiograph, one developed a subphrenic abscess and one an empyema thoracis due to accidental perforation of the oesophagus at operation. The two patients with minor infections showed no change in their βTG concentrations but the five with serious infections showed rises in plasma βTG concentrations coinciding with the development of symptoms or signs of infection. The median βTG concentrations are shown in Fig. 36. This rise became significant by the day symptoms or signs had developed ($p < 0.05$, Wilcoxon). A further six patients showed evidence of minor wound sepsis with some induration and erythema of the wound but no systemic upset. This was not associated with elevated βTG concentrations.

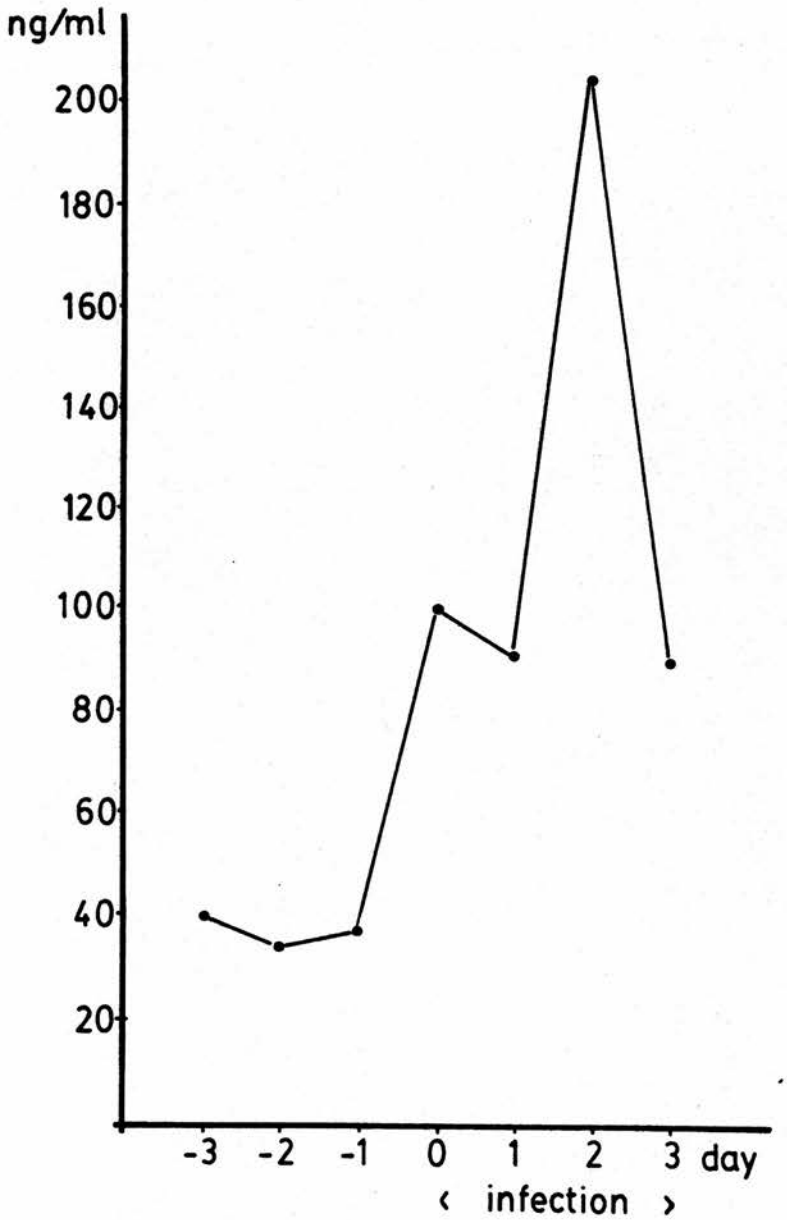

Fig. 36: Median plasma βTG concentrations in 5 patients with severe infections.

vi) *Effect of DVT prophylaxis*

Four standard methods of DVT prophylaxis were used in this survey. One group received intermittent pneumatic calf compression with Roberts pumps during operation; a second group received Dextran 70 in a dose of 500 ml during operation with a second dose of 500 ml within 8 hours of completing the operation; the third group received subcutaneous heparin in a dose of 5,000 units 8 hourly, starting 2 hours before surgery and continuing for 1 week after operation; the fourth group wore graduated compression stockings throughout their hospital stay and the fifth group received no prophylaxis. The incidence of DVT is summarised in Table 20.

Of the GS patients who had no post-operative complications, six received Dextran 70, one received subcutaneous heparin, two received pneumatic calf compression and seven received no prophylaxis. For analysis I have included those receiving calf compression which I regard as unlikely to affect platelet function, with those receiving no prophylaxis and compared them to those receiving Dextran 70. There was no significant difference in βTG concentrations on any of the post-operative days between the groups ($p > 0.1$, Wilcoxon).

Subcutaneous heparin became the method of prophylaxis in our PVD patients. One patient received a continuous heparin infusion instead. Eleven PVD patients who received post-operative heparin and developed no complications have been compared with seven PVD patients who received no prophylaxis. Although the median βTG concentration for the first 4 post-operative days was higher in those

TABLE 20: METHOD OF DVT PROPHYLAXIS RELATED TO INCIDENCE OF POST-OPERATIVE DVT

Group	Method	$^{125}_{IFT}$ Negative	$^{125}_{IFT}$ Positive
GS	Pneumatic compression	4	1
	Dextran 70	28	4
	Subcutaneous heparin	1	2
	TED stockings	1	2
	No prophylaxis	14	5
PVD	Subcutaneous heparin	18	8
	Intravenous heparin	1	0
	No prophylaxis	9	3
	Total	76	25

who received heparin (60 ng/ml) than in those who did not (43 ng/ml), the difference was not significant ($p > 0.1$, Wilcoxon).

vii) *Wound haematoma*

Four PVD patients developed large wound haematomata but this did not produce any detectable change in plasma βTG concentration.

Discussion

The results in the GS patients have been analysed separately from the PVD patients as it has been demonstrated in sections 1 and 2 of this chapter that the release patterns are slightly different for the two groups. Although both groups had a similar age distribution there was a preponderance of females in the GS group but a preponderance of males in the PVD group.

A consistent feature in those whose post-operative progress was uncomplicated was the tendency for the plasma βTG concentration to fall initially after operation only to rise again, peaking on day 4 in the PVD group and day 6 in the GS group. The parallel rise of the βTG/platelet ratio indicates that this was not due purely to the well recognised rise in platelet count which follows operation.[39,153] However, during this period there is a concomitant rise in platelet adhesiveness which has been attributed to an increased proportion of juvenile platelets.[153] After acute blood loss in dogs there is an increased proportion of large heavy platelets[125] and Karpatkin[97] regards large heavy platelets as juvenile. He has also shown that they are more active and will release more PF_4 than small light platelets.[98] The elevated βTG concentrations may, therefore, be a

manifestation of these active, high secreting platelets. Also during this period the process of wound healing is continuing and this is associated with a proliferation of small blood vessels[193] as the granulation tissue develops. These vessels are initially small and fragile and this may be associated with some platelet activity contributing to the rise in βTG concentration.

In the early post-operative specimens (days 1 and 2) the βTG level tends to be lower than even the pre-operative concentrations. This implies a failure of release for which there are two possible causes. A platelet refractory state has been described after operation by O'Brien[145] and after emotional stress by Haft and Arkel.[71] This will, therefore, reduce the extent of the normal continuing platelet activity.

Other workers have also demonstrated that platelets which have released in response to small doses of thrombin or plasmin may have a normal survival and function.[30,163] If the massive release seen during operation (section 2 of this chapter) is release of this type without aggregation there may be a high proportion of platelets with low granule content. Aggregation of these platelets would be associated with much less βTG release. However, the patients who showed little release during operation also had low concentrations in the early post-operative period suggesting that these patients had platelets that were relatively resistant to aggregation.

Various other factors may affect the βTG concentration after operation. Infusion phlebitis produces a small but insignificant rise but blood transfusion has a more pronounced effect. Numerous

drugs were given in the post-operation period but it was difficult to associate particular drugs with βTG release. Heparin has been shown to have an effect on βTG release (Chapter I, section 5) and patients receiving heparin did tend to have a higher βTG concentration. Dextran, however, despite its known effect on platelet adhesiveness,[44] release and aggregation[26] and factor VIII activity,[13] produced no detectable change in βTG concentration when given on the day of operation.

The other major effect on βTG concentration in patients without thrombotic disease appears to be infection. Whilst minor local sepsis had little effect, major infections produced significant release. The release coincided with the development of symptoms or signs. There are several mechanisms by which this effect may be produced. Tissue necrosis may result from serious infections[66] and this will be associated with thrombosis and βTG release. However, infection has a more direct effect on platelets as demonstrated by Reimann as long ago as 1924.[162] He showed a marked fall in platelet count during the fever of pneumococcal pneumonia with a rise following the 'crisis'. In a study of human and porcine platelets, Movat *et al.*[129] showed that the phagocytosis of antigen-antibody complexes could result in platelet release and aggregation. Further studies on bacterial toxins, especially staphylococcal toxins, have shown that they can produce platelet aggregation directly.[15,114]

This study shows that many factors affect the βTG concentrations in convalescent surgical patients of which the most significant non-thrombotic factor appears to be major sepsis. The patients, however, showed widely varying βTG concentrations even in the absence

of an obvious cause. This will severely limit the value of the βTG concentration in the diagnosis of thrombotic disease.

4. THE PREDICTION AND DIAGNOSIS OF POST-OPERATIVE DEEP VENOUS THROMBOSIS

Aim:

To study the value of plasma βTG as a diagnostic or predictive test of post-operative DVT.

Methods:

The patients identified in parts 2 (the study of intra-operative βTG concentrations) and 3 (the study of post-operative βTG concentrations) who developed positive ^{125}IFTs were separately analysed to assess the value of the βTG concentration in i) predicting the development of post-operative DVT and ii) diagnosing post-operative DVT.

Results:

i) *Prediction of post-operative DVT*

Seven patients were identified in the intra-operative study who developed DVT. Six underwent major arterial reconstructions and one a colectomy. There was no difference in intra-operative βTG between those who did and those who did not subsequently develop DVT.

ii) *Diagnosis of post-operative DVT*

General Surgical patients. Fourteen patients developed positive ^{125}IFTs from 1 to 6 days after operation (mean 3.8 days, SD1.8 days) and on the day of presentation 1 to 12 points (mean 4.3 points, SD 3.9 points) on the scans showed increased uptake. The thrombi continued to extend over periods up to 6 days (mean 2.8

days, SD 2.0 days) to a maximum extent of 1 to 22 positive points (mean 9.4 points, SD 7.4 points). Active therapy, depending on the site of the thrombus, was commenced as soon as the diagnosis was made. Patients with thrombi confined to the calf were normally treated by support and mobilisation but those with thrombi extending to the popliteal vein or above received heparin therapy. The incidence of DVT related to age is shown in Fig. 37.

There was no correlation between the plasma βTG concentration and the number of positive points on the ^{125}IFT on the day the test became positive ($r = 0.11$) or on the day the thrombus reached its maximum extent.

If the four patients with complications (3 infection and 1 blood transfusion) are excluded, the median βTG concentrations rose slightly on the day before the ^{125}IFT became positive (Day -1), reached a peak on the day the scans became positive (DVT day) and fell thereafter (Fig. 38). Although the βTG concentrations on Day 1 and DVT day were not significantly different from any of the other post-operative days, they were significantly higher than the pre-operative levels in the same patients ($p < 0.05$, Wilcoxon). There was no progressive rise or even maintenance of the levels in those patients whose DVT extended.

Arterial Surgical patients. The incidence of DVT related to age is shown in Fig. 39. The 11 patients showed similar patterns of onset and progression as the General Surgical patients and again showed poor corelation between plasma βTG concentration and extent of the DVT at the time of onset ($r = 0.18$) or at its maximum extent

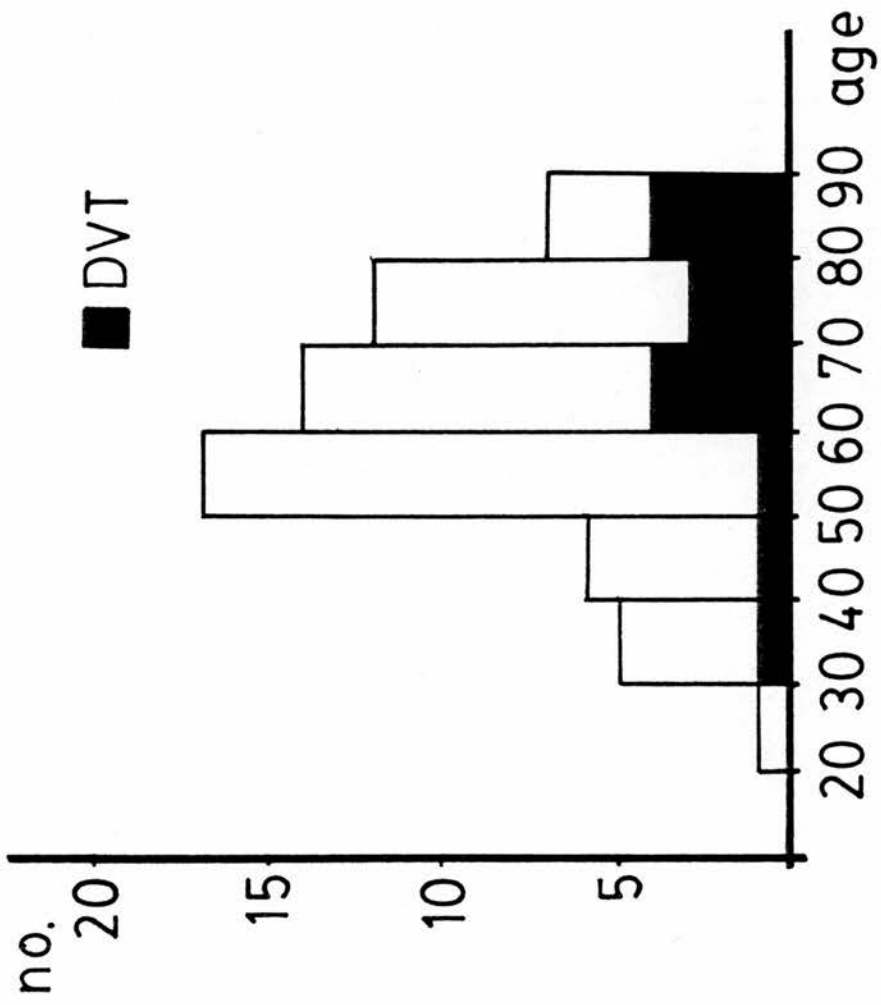

Fig. 37: Incidence of DVT by age in general surgical patients.

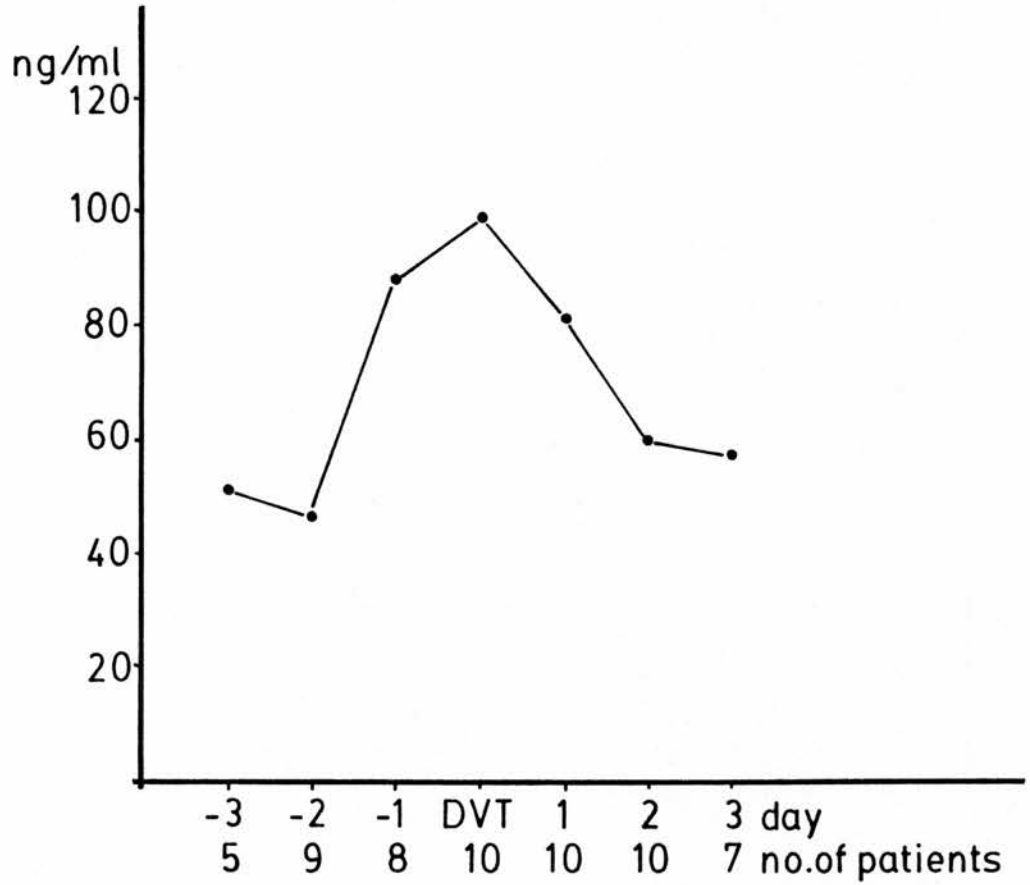

Fig. 38: Median βTG concentrations in 10 patients with DVT following general surgical operations related to the day the ^{125}I fibrinogen uptake test became positive.

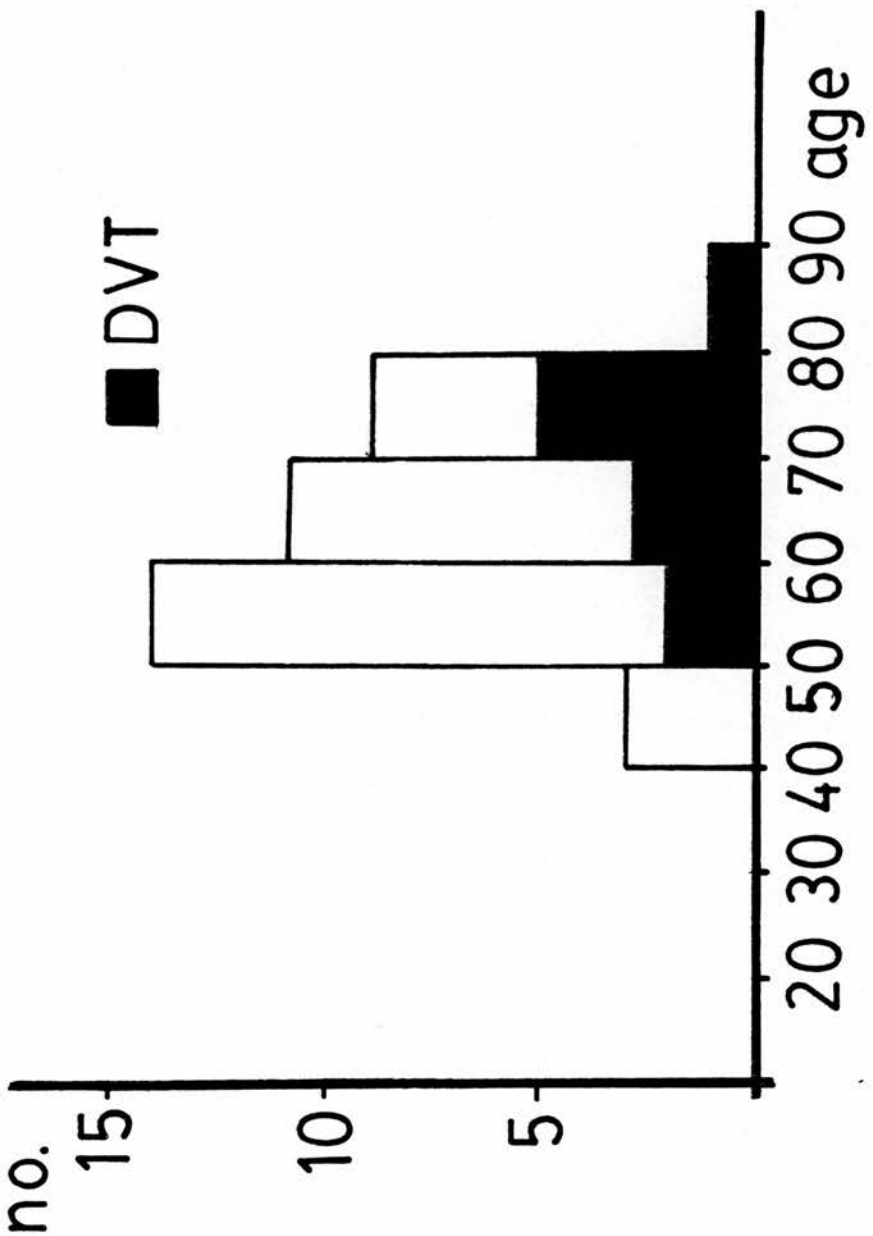

Fig. 39: Incidence of DVT by age in arterial surgery patients.

($r = 0.13$). The median βTG concentrations, including two patients with wound haematomata and five patients receiving subcutaneous heparin, are shown in Fig. 40. There was no rise detectable in relation to the onset of the DVT and none of the days showed concentrations significantly different from the pre-operative levels.

Discussion

Intra-operative βTG concentrations were of no value in the prediction of post-operative DVT. The effects of other factors on βTG during operation would mask any detectable change. Nicolaides[139] has shown that 45% of leg scans were positive on the day of operation and a further 43% within the first 4 days. However, in the present study only 3 of 25 patients with DVT had developed positive ^{125}IFTs within 1 day of operation and, therefore, one would be unlikely to find DVT-related changes in plasma βTG during operation.

There appeared to be no correlation between the extent of the DVT at the time of onset or at its maximum extent and the plasma βTG. As these patients had considerable βTG release in relation to their post-operative state, the additional βTG released by the thrombus will not be in a sufficient excess to render close correlation.

Although the plasma βTG did tend to rise about the time the ^{125}IFT became positive, it was insufficiently reliable to be of any diagnostic value. It is interesting to note that the plasma βTG rose on the day before the ^{125}IFT became positive suggesting that the former was indicating the earliest phase of thrombogenesis and

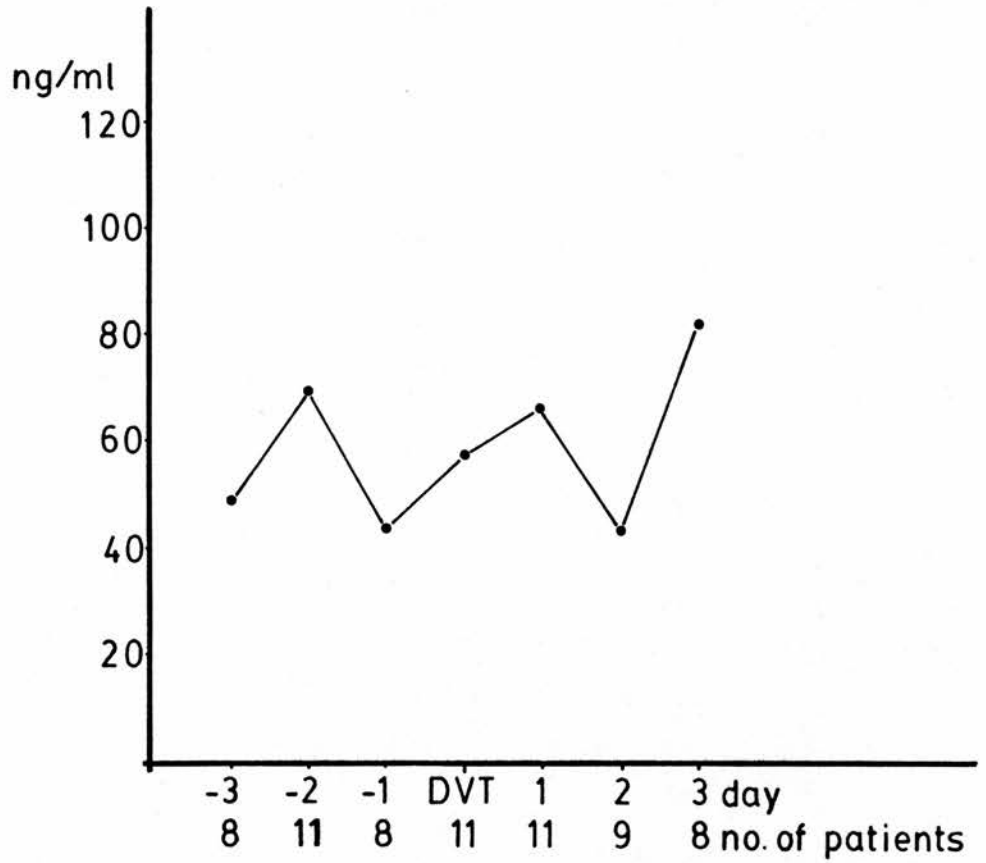

Fig. 40: Median plasma βTG concentrations in 11 patients with DVT following arterial surgery related to the day the ^{125}I fibrinogen uptake test became positive.

that the sensitivity of the test may be potentially better than the ^{125}IFT for early thrombus formation. There was no correlation in the PVD patients and this probably reflects the rather higher βTG concentrations in these patients post-operatively.

A major criticism of the study is that the ^{125}IFTs were only performed on alternate days. However, for a short period additional funds were available to allow daily scanning. Thirty-one patients were, therefore, examined daily and six developed DVT. The correlation between positive ^{125}IFTs and plasma βTG in these patients was no better.

Conclusion:

The plasma βTG concentration appears to be a sensitive indicator of platelet activity but is so lacking in specificity in the post-operative period as to be valueless in the diagnosis or prediction of DVT.

5. THE PREDICTION AND DIAGNOSIS OF POST-OPERATIVE ARTERIAL THROMBOSIS

Aim:

To study the value of plasma βTG as a diagnostic or predictive test of thrombosis of a graft or endarterectomised segment following reconstructive arterial surgery.

Methods:

Four patients in the major arterial surgery group occluded their grafts or endarterectomised segments within 24 hours of operation. Thrombosis was diagnosed by the loss of a restored pulse or the deterioration in clinical state of the limb following reconstructive operation.

Results:

Scrutiny of the intra-operative and post-operative plasma βTG concentrations failed to reveal any significant difference between the four patients who thrombosed their grafts and the 11 others whose progress was uncomplicated. The levels in the four patients with thrombosis tended to be higher but the overlap with the non-thrombotic group was such that the test was not diagnostic. Three of the non-thrombotic group developed major haemorrhagic problems and all of them (as well as one in the group with graft thrombosis) had very high concentrations in the immediate post-operative specimen (> 1,000 ng/ml).

Discussion

Again, the factors other than thrombosis that affect platelets in the peri-operative period render the plasma βTG concentration of no value in the diagnosis or prediction of graft occlusion. However, a plasma βTG concentration in excess of 1,000 ng/ml on the immediate post-operative specimen suggests imminent major thrombotic or haemorrhagic complications. This, however, is of little value as such complications are usually clinically obvious.

6. URINARY BETA-THROMBOGLOBULIN CONCENTRATIONS FOLLOWING MAJOR ABDOMINAL SURGERY

Aim:

To assess the effects of post-operative factors on the urinary βTG concentrations in patients without DVT and so indicate whether further studies of the use of urinary βTG in the diagnosis of post-operative DVT are justified.

Methods:

Six patients were studied following major abdominal surgery. Five had negative ^{125}IFTs and one had a negative phlebogram. In three patients urine samples were obtained every time the patient passed urine from before operation until 3 days afterwards. One patient had an indwelling catheter and samples were obtained every 4 hours. The total volume of each sample and the time it was passed were recorded and an aliquot removed for βTG assay. Plasma samples were obtained at intervals during the urine sampling. The other three patients developed painful calves at 4, 10 and 13 days after surgery and had no evidence of DVT on phlebography or ^{125}IFT. Plasma and urine samples were obtained at the time of the calf pain.

Results:

i) *Population statistics*

Three males and three females aged 36-76 years (mean 66 years, SD 7.5 years) were studied.

ii) *Urinary BTG*

In the three patients intensively screened for 4 days all the pre-operative concentrations were within the normal range. One patient (3) had a urinary catheter inserted immediately after surgery and, although his plasma βTG concentrations following surgery were only just elevated, his urinary concentrations became very high. Inspection of the urine revealed microscopic haematuria presumably associated with the indwelling catheter. One patient (2) without complications developed high urinary βTG concentrations after operation and the other patient (1), who developed a faecal fistula, had normal urinary and plasma βTG concentrations throughout. The plasma and urine levels in patients 1 and 2 are shown in Fig. 41. The three patients (4, 5 and 6) investigated for calf pain 4, 10 and 13 days after surgery all showed elevated urinary concentrations. The data on these patients are summarised in Table 21.

Discussion

The urinary concentrations tended to follow the plasma concentrations in the post-operative period. None of these patients had detectable DVT but they all showed elevated plasma and urine concentrations, except patient 1 who showed no release during or after surgery. Despite the consistently elevated plasma concentrations in patient 2, the urine concentrations showed very marked fluctuations in concentration indicating a very variable platelet activity. The patient with the indwelling catheter showed quite markedly elevated urinary concentrations in the specimens in which blood was detectable.

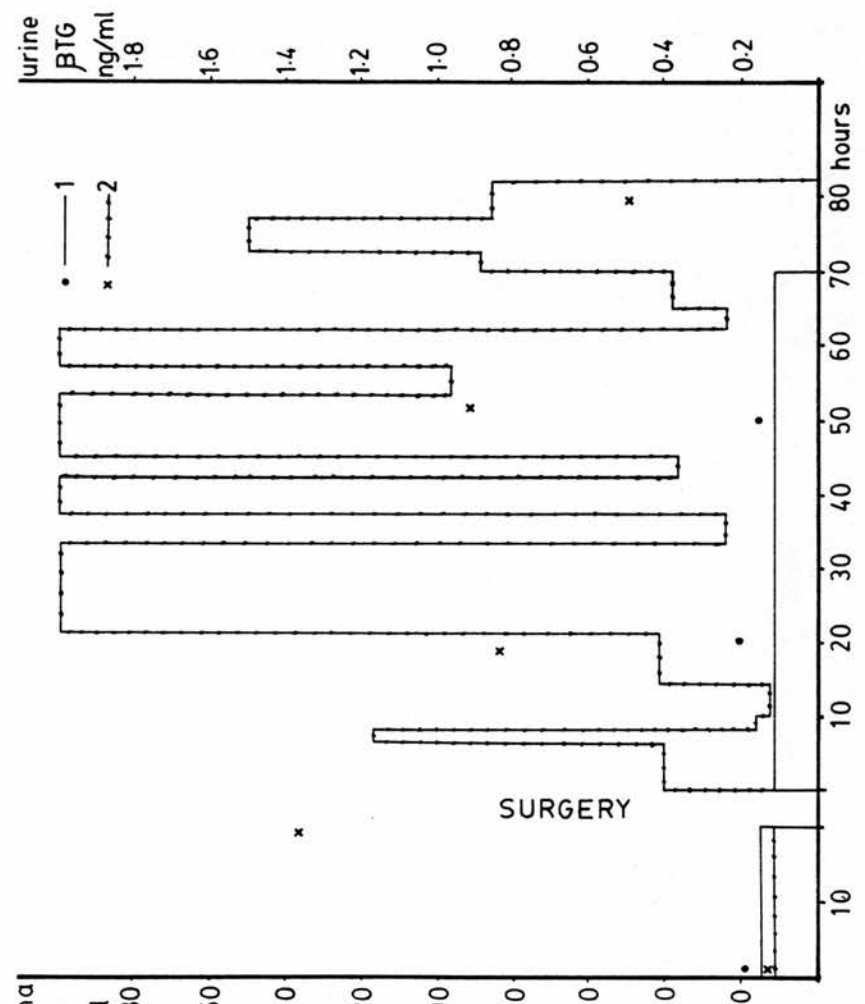

Fig. 41: Plasma and urine βTG concentrations in 2 patients before and after operation.

TABLE 21: PLASMA AND URINE βTG CONCENTRATIONS IN 6 SURGICAL PATIENTS

	Pre-op. βTG (ng/ml)		Post-op. βTG (ng/ml)	
Patient No.	Plasma	Urine	Plasma	Urine
1	19.3	0.15	16.4-200	0.12
2	16-138	0.12	50-91	0.12-2.0
3	162.2	0.15	75-132	0.10-0.89
4	-	-	45-202	0.73-1.81
5	-	-	222.5	0.69
6	-	-	56.4	0.65-0.93

In the patients studied late in the post-operative period the urinary concentrations were still elevated despite some normal plasma concentrations. This is evidence of persisting episodic release continuing in these patients.

Conclusion:

The measurement of urinary βTG, although of value in diagnosing DVT, as I have shown in Chapter II, is beset by a similar lack of specificity in the post-operative period as the plasma βTG concentration.

CHAPTER IV

ARTERIAL DISEASE

INTRODUCTION

The studies described in Chapter III showed that patients with peripheral arterial disease tend to have more persistently elevated plasma βTG concentrations than those without clinical evidence of arterial disease. Why might this be so? There are two possibilities.

1) Atherosclerosis develops as a result of increased platelet activity. In 1946 Duguid[49] suggested that many of the lesions we now classify as atherosclerosis are arterial thrombi which, by the ordinary processes of organisation, have been transformed into fibrous thickenings. More recently, Carstairs[28] and Woolf[204] have been able to demonstrate platelet antigens in fibrolipoid plaques but not in fatty streaks. Atherosclerosis may develop as a result of increased platelet activity and, therefore, patients with more extensive disease may have greater platelet activity detectable by elevated plasma βTG concentrations.

2) Atherosclerosis results in widespread endothelial damage which causes increased platelet activity. A good example of platelet activity resulting from atherosclerosis is the condition amaurosis fugax where platelet emboli pass to the retina from the ulcerated plaque in the internal carotid artery.[61] Those with more extensive disease may be expected to have more evidence of platelet activity. Murphy[133] and Abrahamsen[2] have shown a reduced platelet survival in

patients with atherosclerosis and spontaneous platelet aggregation has also been demonstrated. Ward[194] and Olcott[149] have shown an increased aggregability in response to ADP and adrenaline in patients with more extensive disease than in those with minor forms.

Other authors have already shown some relationship between plasma βTG concentration and arterial disease.[92,119]

If the extent of the atherosclerosis is related to plasma βTG it might be a useful screening test to detect those at risk of major complications in whom appropriate therapy may limit progression.

Aims:

To answer the following questions:

1) Is atherosclerosis associated with evidence of platelet activity?
2) If so, is the βTG level related to the extent of the disease?

METHODS:

Eighty-five consecutive patients attending a peripheral vascular clinic with a complaint of limb pain were studied. Plasma and urine samples were obtained from them in the standard fashion and assays were performed for plasma and urine βTG and PF_4. A platelet count was performed on a portion of the sample. On the basis of the history, physical examination and investigation, the patients were allocated scores for the extent of their vascular disease.

One point was scored for evidence of vascular disease in each of the following areas:-

Carotid or cerebral vessels

Each upper limb

Coronary vessels

Aorta and visceral branches

Each iliofemoral segment

Each leg below the common femoral bifurcation

The maximum possible score was 9 but no patient scored more than 7. This scoring system therefore produced 8 groups of patients 0 - 7.

The criteria of arterial disease were as follows:-

- in a limb - the presence of bruits, the absence of pulses or a reduction in arterial pressure index.
- in the carotid or cerebral circulation - the history of stroke or transient ischaemic attack, the presence of carotid bruits or the absence of carotid pulses.
- in the coronary circulation - a history of angina or proven myocardial infarction or the presence of ischaemic changes on an electrocardiogram.

Patients with aneurysmal disease were excluded and angiographic findings were not considered as few patients were subjected to this investigation.

Group 0 (those without evidence of atherosclerosis) was divided into two sub-groups. The first sub-group (A) was composed of 10 patients who acted as 'controls'. These patients were suffering from limb pain due to predominantly musculo-skeletal disorder such as lumbar spondylosis, osteoarthritis, cervical ribs or peripheral neuropathy and had no evidence of vascular disease. The second

sub-group (B) comprised 10 patients without major arterial disease but who had vascular diseases such as varicose veins or vasospastic disorders.

A record was also made of all the patients' smoking habits, any drugs they were currently receiving and any previous arterial reconstruction.

RESULTS:

i) Population statistics

The incidence of smokers in each group varied from 50% in group 0A to 100% in groups 6 and 7 (Fig. 42).

The mean age varied from 48.7 years in group 0B to 74.7 years in group 3. Again this variation did not reach statistical significance between the control group 0A and any of the other groups. The mean age in group 0B, however, was significantly lower than in groups 3, 4 and 5 (Student's t test, $p < 0.05$).

No patients were on anticoagulants and only two received any drugs known to affect platelet function - one patient in group 7 on Aspirin and one in group 3 on Naprosyn.

ii) Plasma βTG

Three patients - two in group 0A and one in group 2 - had plasma βTG concentrations > 1,000 ng/ml. These have been excluded for reasons explained later. The results have been shown in Fig. 43. There was no significant difference between any of the groups with the exception of between group 2 and groups 1, 0A and 0B (p 0.05, Wilcoxon). However, when those with minimal disease

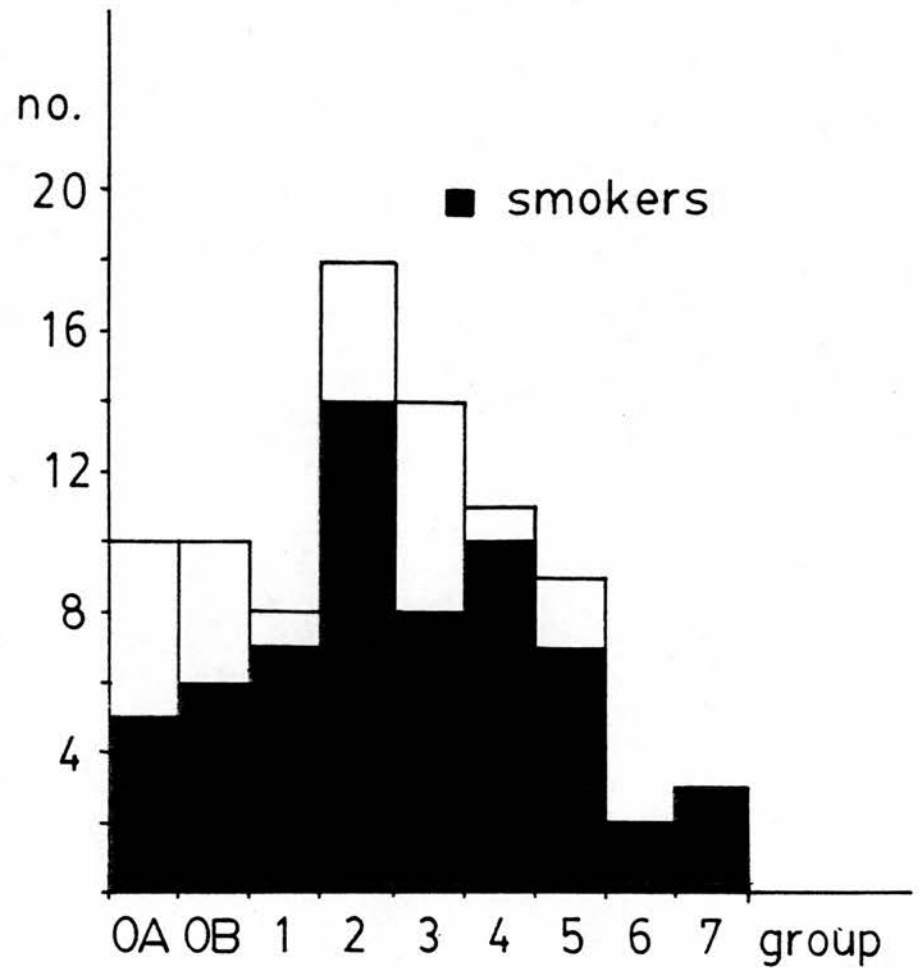

Fig. 42: Smoking and clinical groups in atherosclerotics.

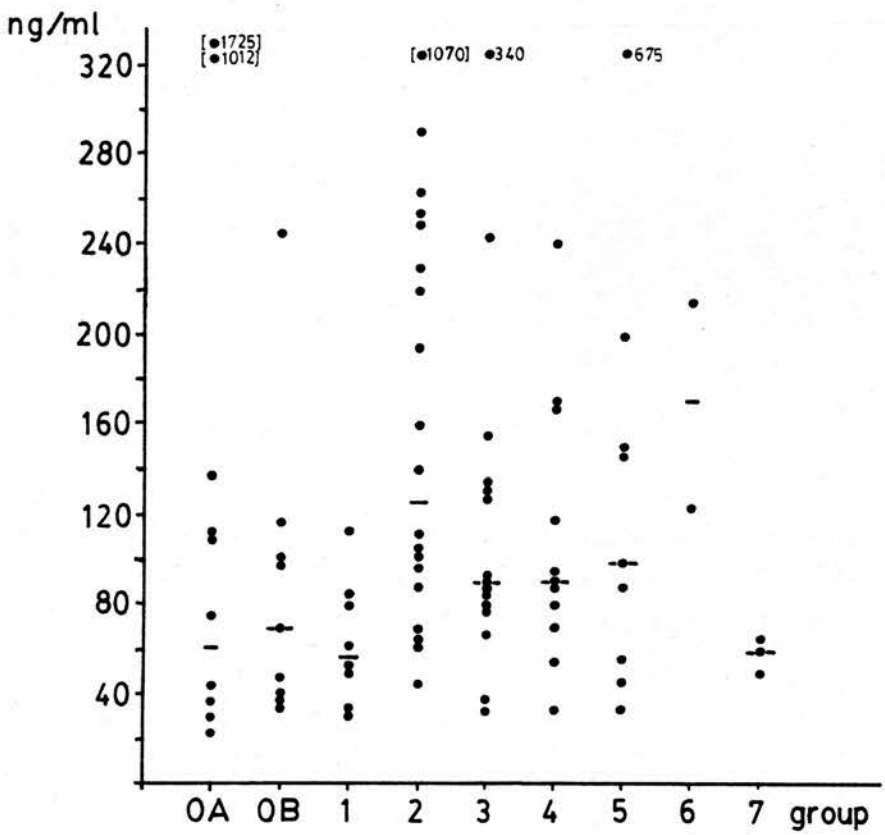

Fig. 43: Plasma βTG concentrations by clinical group in atherosclerotics. Median concentration indicated in each group.

(groups 0A, 0B and 1) were compared with those with more extensive disease (groups 2 - 7), the difference was highly significant ($t = 3.36$, $p = 0.0012$).

Figure 44 shows the proportion of patients in each group who had abnormal βTG concentrations (> 80 ng/ml). This varied from 38% in group 1 to 78% of group 3. There was no significant difference between the groups (χ^2 distribution on 2 x 2 tables, $p > 0.1$), although there was a slight tendency for a higher proportion of the patients with more extensive disease to have abnormally high concentrations compared to the control groups.

The plasma βTG concentrations did not correlate with smoking nor with age ($r < 0.1$) even in groups 0A and 0B.

iii) Plasma PF_4:

The plasma PF_4 results gave similar distributions to those of βTG and did differentiate between those with extensive disease and those with minimal disease. There was good correlation between plasma βTG and PF_4 throughout the study with correlation coefficients varying from 0.43 in group 1 to 0.98 in group 5. The correlation coefficient for the whole series was 0.79.

iv) Urine βTG:

Only six urine samples gave concentrations in excess of 0.21 ng/ml and these were distributed in groups 1 - 5. No abnormal concentrations were detected in groups 0A, 0B, 6 or 7.

v) Urine PF_4: No abnormal levels were detected.

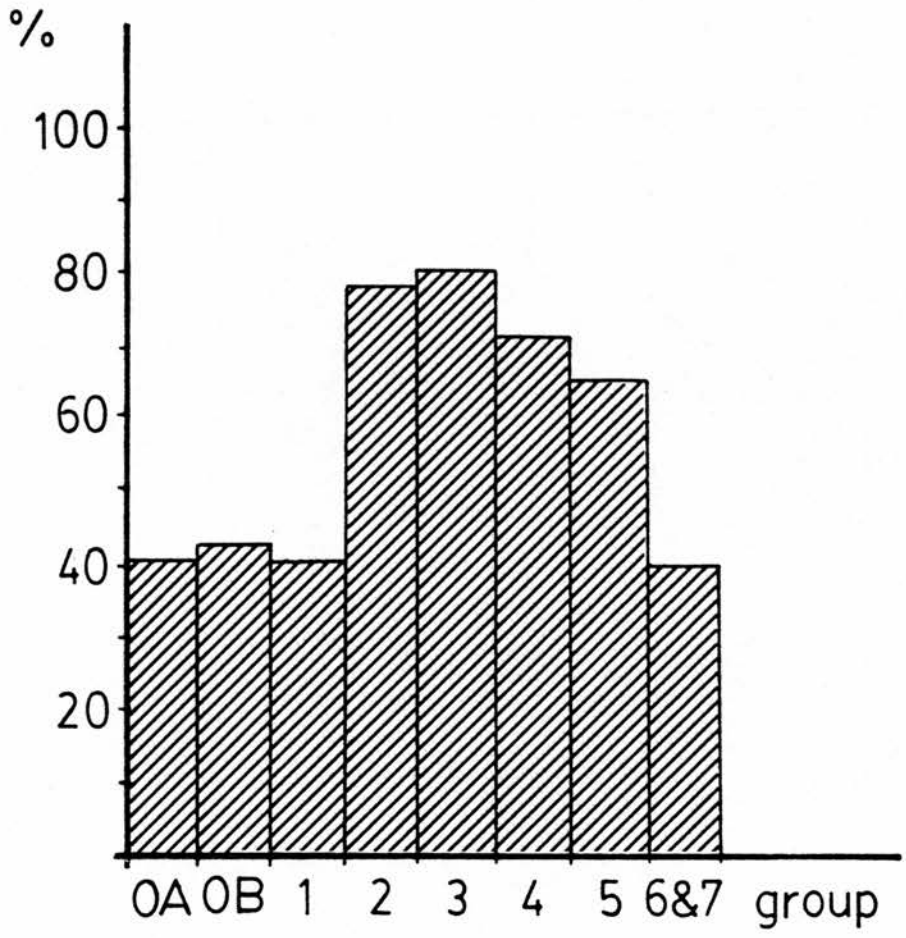

Fig. 44: Incidence of abnormal plasma βTG concentrations (per cent > 80 ng/ml) by clinical group in atherosclerotics.

DISCUSSION

The most difficult part of the study is the quantification of the arterial disease as no satisfactory technique exists at present apart from extensive autopsy dissections. Angiography gives a good impression of the extent of vascular disease but total body contrast radiology is impractical and, in fact, few of our patients required any form of angiography.

Various authors have devised different ways of assessing the extent of arterial disease and some of them have even classified the extent of this generalised disease purely on the clinical findings in one part of the body. Ward[194] gave little indication of how he classified his patients but he seemed to grade them on the basis of clinical examination, the ankle pressure index, presence of myocardial ischaemia and the history of smoking. However, Olcott[149] avoided the issue by classifying them into 'mild' if they had detectable disease at one site and 'severe' if they had detectable disease at more than one site. Cella *et al.*[31] classified the extent of the arterial disease on the claudication distance. Severe claudication can be caused by very localised lesions and this classification, too, seems unsatisfactory.

The classification devised for this study attempts to take account of the generalised nature of the disease, but even so there are major fallacies. It is based, along with the others quoted, on the clinical consequences of significantly reduced blood flow. However, one would expect platelet activity to be related to the surface area of abnormal endothelium and in patients with

atherosclerosis there may be extensive endothelial damage without significant alterations in blood flow. This classification depends on the assumption that larger areas of endothelial damage are present in patients with tight stenotic lesions or occlusions.

Another problem with the classification is the interpretation of the electrocardiogram. ST and T wave changes are entirely non-specific and were discounted unless they represented a change from a previous ECG. The site of a lesion is also important. A minor lesion in a cerebral vessel may produce a major clinical event whereas a much more extensive lesion in the lower limb may be entirely asymptomatic. This system of classification is, therefore, a compromise.

The plasma assays in three patients have been excluded from the analysis - two in group 0A and one in group 2. All three had βTG concentrations of 1,000 ng/ml or greater and are grossly different from the other concentrations in the same groups. All three samples were processed in the same batch and it is assumed that there has been a technical error in the processing of the specimens.

There did tend to be higher βTG concentrations associated with more extensive disease and this reached significance for groups 2 - 7 compared to groups 0A - 1. Less than the 50% of the patients in groups 0A, 0B and 1 had abnormal βTG concentrations whereas groups 2 - 5 showed elevated concentrations in over 60%. There were insufficient patients in groups 6 and 7 to allow valid analysis. The plasma βTG concentrations did, therefore, tend to be higher in

those with scores 2 and above than in those with minimal disease.

The 40% incidence of abnormal βTG concentrations in the control groups (0A and 0B) is much higher than in the normal range. However, the age range in group 0A, although older than the normal group, was similar to the atherosclerotic patients. Ludlam[109] has shown that βTG tends to rise with age and this may be a manifestation of subclinical atherosclerosis. Group 0B patients were younger but they did have other vascular diseases (acrocyanosis and Raynaud's disease) which may be associated with platelet activity. I was unable to correlate the plasma βTG concentration with age unlike Ludlam.[109] This may be due to the extensive vascular disease present in many of these patients.

Only 7% of patients had abnormal urinary *βTG* concentrations suggesting that the platelet activity detected by the plasma βTG concentrations is not sustained. Although significantly elevated concentrations can be detected in patients with extensive disease, some patients had normal concentrations. This implies that the activity is episodic and insufficient to produce elevated urinary concentrations. The DVT patients, although having similar plasma concentrations, have more consistently elevated urinary concentrations indicating a more continuous platelet activity.

The wide scatter of abnormal plasma concentrations detected throughout the study show either that the classification bears no relationship to platelet activity or that the classification is not a true measure of the severity or extent of the disease. The study, in fact, may have been dealing with patients who had similar areas of

abnormal endothelium despite widely varying clinical presentations.

Cella *et al.*[31] found a significantly increased plasma βTG in a group of patients with peripheral vascular disease compared to controls but only a third of the patients had elevated concentrations. Their controls, however, were healthy and were approximately half the age of the patients. Zahavi and Kakkar[209] were able to relate the magnitude of the rise of βTG to the ankle systolic pressure index. Both these studies do not seem to relate the plasma βTG to the true extent of the arterial disease. Zahavi, in fact, may be correlating the rise in plasma βTG to the extent of ischaemic tissue.

Another aspect of the platelet-atherosclerosis relationship is the role of the platelet in the development of the atherosclerotic lesions. Woolf[204] was able to demonstrate platelet antigens using antiplatelet globulin in atherosclerotic plaques and it may be that atherosclerosis is a late manifestation of mural platelet thrombus. Enhanced platelet aggregability, reduced survival or elevated plasma βTG may be an aetiological factor in atherosclerosis. However, as we know there is extensive platelet activity on mature atherosclerotic plaques, it is impossible to differentiate between causative and resultant platelet abnormality. Only a prospective study of patients as they develop atherosclerosis could solve that problem and it is beyond the scope of this study.

All that can be concluded from this study is that patients with extensive symptomatic atherosclerosis tended to have higher plasma βTG concentrations than those with less extensive disease. No comment can be made as to whether this finding is of aetiological significance.

CONCLUSIONS

THE INFLUENCE OF SAMPLING METHODS:

1. The addition of prostaglandin E1 to the combination of EDTA and theophylline added little to the stability of normal platelets but it was of value in the samples from patients with DVT. Even so, the period of platelet stability in this mixture of three chemicals was only about 24 hours at 4^0C. In this study the plasma was always separated within 12 hours and usually within 3 hours.

2. Adding the anticoagulant mixture to the syringe did not significantly affect the plasma βTG concentration but it did eliminate the spuriously and inexplicably elevated results which occurred occasionally throughout the study. A possible explanation of these high results has been provided recently by Rasi[160] who showed that there may be enrichment of the top third of the plasma by platelet debris after centrifugation. He now recommends sampling the middle third of the plasma supernatant whereas we sampled the top third throughout the study. The syringe tests showed that little release occurs until at least 4 mins have elapsed and most samplings are completed in no more than 1 min.

3. Venous stasis for the purpose of blood sampling has no effect on plasma βTG, nor does the presence of indwelling catheters. Sampling through an indwelling catheter however is unreliable due to platelet release in the catheter system.

4. Because the distribution of βTG concentrations in normals was grossly skewed, the normal range was arbitrarily set at a level to

include 95% of the samples. This gives an upper limit of normal for plasma βTG of 80 ng/ml and for urinary βTG concentrations of 0.21 ng/ml. The Radiochemical Centre RIA Kit was consistently less sensitive than the local MRC radioimmunoassay. The βTG excretion rate in normals correlated well with the urinary βTG concentration but less well with the urine flow rate. Measurement of urinary βTG concentration alone gives a good indication of the rate of βTG excretion.

5. Following the infusion of a βTG load the clearance of the plasma βTG is closely followed by its appearance in the urine. Following an initial phase of redistribution it is cleared from the plasma with a half life of about 80 mins. Only about one fifty-thousandth of the infused dose appears in the urine.

6. The correlation between plasma βTG concentration and platelet count was poor at normal platelet counts but much better in thrombocytopenic patients. There is no need to correct plasma βTG for platelet count unless the platelet count is unduly low. There was no relationship between plasma and serum concentrations of βTG.

7. Aspirin had no effect on plasma βTG concentration or on the syringe test in contrast to Heparin which was often followed 6-8 hours after injection by βTG release. The interpretation of plasma βTG concentrations in patients on heparin therapy may therefore be unreliable.

VENOUS THROMBOEMBOLISM:

1. The plasma βTG concentration was of little value in the diagnosis of venous thromboembolism. Only 63% of patients with DVT had raised concentrations and 44% of those without had a positive result. This test does not reach a useful level of diagnostic accuracy. The urine βTG concentration is a more valuable test as 95% of those with DVT, but only 28% of those without, had elevated concentrations. A normal concentration virtually excludes a diagnosis of venous thromboembolism but a raised concentration indicates the need for further more specific investigations such as phlebography. This suggests that the measurement of the urine βTG concentration would be a useful preliminary screening test in patients suspected clinically of having venous thromboembolic disease.

2. Syringe tests on patients with acute DVT showed rapid release patterns indicating a higher population of more active platelets.

PERIOPERATIVE PATIENTS:

1. Admission to hospital is associated with βTG release in about a third of patients. The operation produces a marked rise in βTG concentration depending on the extent of the surgical trauma. The more extensive procedures - particularly arterial reconstruction - tended to have higher levels and more prolonged periods of raised plasma βTG concentrations.

2. Following operation the plasma βTG concentration fell initially but rose again to peak about days 4 - 6. Infusion phlebitis, blood

transfusion and the use of subcutaneous heparin produced small rises in plasma βTG concentration. Minor infections had little effect but markedly elevated concentrations were found in patients with major sepsis.

3. Post-operative DVT diagnosed by the ^{125}IFT was associated with a small rise in plasma βTG but this was not diagnostic. Plasma βTG also failed to predict or diagnose the thrombosis of arterial reconstructions.

4. Urine βTG concentrations were affected by the same factors as plasma βTG and hence were of no diagnostic value.

ARTERIAL DISEASE

The plasma βTG concentrations could not be correlated with age but patients with more extensive arterial disease detected clinically tended to have higher concentrations. Some patients without clinically detected arterial disease but with other conditions also had elevated βTG concentrations. Very few patients had elevated urinary concentrations.

THE FINAL CONCLUSIONS OF THIS STUDY ARE:

1. The plasma βTG concentration is a sensitive indicator of platelet release and may be of value in carefully controlled experimental situations.

2. The plasma βTG concentration is of no value clinically in the diagnosis of venous or arterial thromboembolic disease.

3. The urinary βTG concentration may be of use clinically as an initial screening test for patients suspected of having venous thromboembolism.

A P P E N D I C E S

APPENDIX A

^{125}I FIBRINOGEN UPTAKE TEST

On the day prior to operation the patient was given 110 μCi of ^{125}I fibrinogen (Radiochemical Centre, Amersham) intravenously. The patient's legs were then marked with indelible crosses at 5 cm intervals from the posterior aspect of the knee joint down the middle of the back of the calf to the ankle and at 5 cm intervals up along the medial aspect of the thigh in the line of the femoral vein to within 10 cm of the inguinal ligament. The first scan was performed later the same day.

Using a Pitman Ratemeter the maximum precordial count was obtained and the ratemeter was set to record this as 100%. With the patient's legs elevated to 30^0 - 40^0, counts were obtained at the marked points on each leg and recorded as a percentage of the maximum precordial count.

The scans were repeated on day 1 after operation and on alternate days thereafter. If the scan became positive, daily scanning was performed.

A scan was classified as positive if it fulfilled one or more of the following criteria maintained for at least 24 hours:

1. a rise of 20 percentage points relative to the same area on the pre-operative scan.
2. a rise of 20 percentage points relative to the adjacent areas.
3. a rise of 20 percentage points relative to the same area on the opposite leg on the same day.

Scanning continued until day 11 or until the day the patient was discharged from hospital.

The thyroid was blocked with iodide - potassium iodide, 60 mg t.i.d. orally or sodium iodide, 100 mg b.d. intravenously - before and during the period of scanning.

APPENDIX B

THE RADIOIMMUNOASSAY FOR BETA-THROMBOGLOBULIN

Venous blood was collected using 21 gauge needles and polypropylene syringes and 2.7 ml were transferred immediately to a siliconised glass tube containing 0.1 ml of 10% EDTA, 0.1 ml of 1 μg/ml prostaglandin E_1 and 0.1 ml of 30 mM theophylline maintained below 4^0C in melting ice. After mixing and within 12 hours the sample was centrifuged at 1,900 *g* for 1 hour at 0^0C and the top third of the platelet poor plasma removed for assay.

Aliquots of plasma (0.01 ml) were taken and diluted to 0.2 ml with 0.05 M sodium phosphate buffer, pH 7.5, containing 2% horse serum. ^{125}I labelled purified βTG was added in 0.05 ml of diluent followed by 0.25 ml sepharose-coupled rabbit anti-βTG antiserum at a dilution to bind about 50% of tracer. The tubes were mixed gently for 1 hour; the antibody-bound protein was separated from the free βTG by centrifugation and the ^{125}I in the residue (antibody-bound fraction) was measured.

Using graphs prepared from standard concentrations, the concentration of βTG in the specimen could then be calculated.

APPENDIX C

STATISTICAL ANALYSIS

All statistical analyses were performed with a 'Commodore' pre-programmed statistical calculator.

Data suitable for analysis with a 2 x 2 table were assessed by the χ^2 test using Yate's correction. If the total of the table was < 20 or any individual cell had < 5, the Fisher Exact test was used.

As most of the data on βTG concentrations did not conform to a normal distribution, the median has been used throughout as a summary statistic. Analysis was by the non-parametric Wilcoxon Rank Sum tests. If the numbers in the groups exceeded 25 x 50, the groups were 'normalised' by conversion to their natural logarithms and then analysed by Student's t test. Much of the population data did conform and was assessed by the t test without prior 'normalisation'.

Correlation was assessed by the standard r coefficient which assesses how closely the data corresponds to a straight line.

APPENDIX D

REVIEW OF THE LITERATURE ON BETA-THROMBOGLOBULIN 1980-1983

Further investigations have been reported on the mixture used to stabilise the blood before plasma separation. Randi *et al.*[235] reported their comparison of EDTA and Theophylline with the combination including Prostaglandin E1. The mean value and the range were significantly greater when the Prostaglandin was omitted. They also commented on the value of the ratio of βTG to PF_4. As PF_4 is very rapidly cleared from the circulation a rise in sample PF_4 is more indicative of *in vitro* release. Therefore a high βTG:PF_4 ratio indicates *in vivo* rather than *in vitro* release. Contrary to expectation they found a higher ratio without the Prostaglandin than with it but this did not reach statistical significance. Franchi *et al.*[224] also confirmed the findings of this study by showing that ETP produced significantly lower values than ET alone. In a study of blood at room temperature, Turnbridge *et al.*[240] showed that EDTA enhanced platelet release and that citrate was marginally better at preventing release. Prostaglandin E1, however, successfully stabilised the blood for up to 3 hours. They do suggest that a more concentrated solution of prostaglandin be used (2×10^{-6}M or 2×10^{-4}M).

Further comment on the βTG:PF_4 ratio was made by Arocha-Pinango.[212] They showed occasional very high or very low ratios in normals which further confirms the finding of this study of occasional spurious results. They found the highest incidence of abnormal ratios in pre-eclamptic patients and also in the puerperium. This suggests that these patients have a high *in vivo* release as well as having platelets which release very readily when handled. This could be due

to the increased number of young, active platelets at these times. Kaplan and Owen,[229] in a review article, make the very valid comment that studies of the clearance of βTG and PF_4 after serum infusions may be affected by platelet release induced by thrombin in the serum. To overcome that problem the blood was processed in plastic bags to which the majority of the thrombin adheres. The thrombin is also rapidly cleared from the circulation so that any effect would be short-lived. If it did engender further release *in vivo*, this should not significantly affect the clearance rate as it is assessed on the plasma concentrations after the initial redistribution period of about 30 minutes. By that stage any effect of the thrombin should have passed.

An extensive study of the effect of membrane active drugs on the alpha granule release was reported by Prowse *et al.*[233] This project was stimulated by the marketing of a new platelet stabilising anticoagulant containing Procaine (Thrombotect) which permitted room temperature processing. They tested a wide range of membrane active drugs including local anaesthetics, anti-malarials, beta-blockers, anti-anginals, anti-inflammatories and anti-helminthics. With increasing concentrations of the drugs, the initial membrane stability induced changes to a state of membrane instability and rupture. Many of the drugs which prevented aggregation did not prevent alpha granule release. Procaine and the anti-malarials, such as hydroxychloroquine, prevent βTG and PF_4 release at 20^0C and therefore, if the blood is stored at 0^0C, it can be processed at room temperature. This avoids the need for a cooled centrifuge.

Cella and Girolami[216] studied the effect of different types of heparin on platelet release. They confirmed the marked release of PF_4 which clears within an hour but failed to show any βTG release. However, they only sampled for two hours and so may have missed the late βTG rise. They also looked at the effect of heparin on aggregation and showed that, although adrenaline induced aggregation was reduced, the response to collagen and ADP was enhanced. This could possibly explain the late βTG rise seen in the present study. The reason for the marked PF_4 release with heparin remains controversial. Kaplan and Owen[229] report that heparin does not release PF_4 *in vitro* and suggest that the PF_4 is released from endothelial binding sites. However, the process of preparation of the platelets for *in vitro* studies may itself affect the platelets and prevent them releasing the PF_4. This would give a spurious impression of lack of response to heparin.

Several groups have also studied the effects of Aspirin and all of them have used more prolonged courses of Aspirin therapy than the single dose used in our study. Santos *et al.*[238] found that, after receiving Aspirin in a dose of 1g/day for three days, platelets from healthy subjects showed a 31% reduction in βTG release following mechanical stimulation by stirring. There was no effect on plasma βTG. Collagen and thrombin produced normal βTG release whether or not the platelets aggregated. Hoogendijk *et al.*[226] were also unable to demonstrate any change in plasma βTG after Aspirin in a dose of 200 mg three times a day for one week in healthy subjects. These findings correlate with the results of the present studies. There was no effect on the plasma βTG nor on the syringe test which is based on

thrombin induced release.

In contrast, Viero *et al.*[245] studied the effect of Aspirin in abnormal platelet populations in patients with myeloproliferative disorders. After Aspirin in a dose of 500 mg/day for 5 days they showed that there was a reduction in plasma βTG but that it did not reach normal levels. This indicates that Aspirin seems to have a greater effect on abnormal platelets than on normal ones; a property of therapeutic interest.

Further studies on the value of βTG in the diagnosis of venous thromboembolism have also been published. In 39 patients suspected of having a DVT and 37 patients with symptoms suggestive of pulmonary embolism, van Hulsteijn *et al.*[242] quote a remarkable 100% sensitivity and 96% specificity for the plasma βTG assay. The 26 patients with proven DVT had had symptoms for up to three days and in 5 patients the thrombus was confined to the calf. This is in marked contrast to the 71% sensitivity and 56% specificity in our study in a similar group of patients. They also found raised plasma βTG concentrations in patients with septic arthritis, rheumatoid arthritis and malignancy but found that diminished renal function did not affect it. A study of 36 patients after total hip replacement by Lane *et al.*[230] showed that all patients had elevated plasma βTG. The 13 who developed DVT had slightly higher concentrations but this did not reach statistical significance. de Boer *et al.*[218] found the assay to be less reliable than van Hulsteijn in the diagnosis of 52 patients with DVT. They found the sensitivity of the plasma βTG to be 35% and the specificity to be 80% but with the urine assay they

had a sensitivity of only 37% but a specificity of 100%. These latter urine results contrast slightly with the sensitivity of 95% and the specificity of 72% of our study. However, they also comment that a third of patients, both with and without DVT, showed considerable variations from day to day. The diagnosis of DVT after elective operation in 36 patients using the βTG concentration of an early morning urine specimen was investigated by Bolton *et al.*[215] They found a specificity of 83.3% and a sensitivity of 87.5% for the test. However, they did find that 92% of patients had a rise in βTG after operation. By the third post-operation day 94% of these raised concentrations had returned to normal. In the present study the post operation urinary βTG sampling was discontinued after 3 days as the results were variable. If sampling had been continued in this group, some useful data may have been obtained.

βTG passes into most body fluids including saliva and Cooke *et al.*[217] report on a rather novel approach to the diagnosis of post-operation DVT. They assessed the salivary βTG in 10 patients following hip surgery and showed that the 6 patients who developed DVT all had markedly elevated βTG concentrations whereas 3 of the 4 without DVT had no rise. This technique has the potential benefit of avoiding the problem of *in vitro* release but could well be affected by minor local pathology in the mouth such as ulceration.

A follow-up study on plasma βTG concentrations after venous thromboembolism in non-surgical patients was reported by van Hulsteijn *et al.*[241] who found that all patients had elevated plasma βTG on admission, only half still had elevated concentrations at 10 days and all had returned to normal by one year.

Farrell *et al.*[222] report an interesting study on lung diseases diagnosed by ventilation and perfusion scanning. They found that the plasma βTG concentrations were similar in patients with pulmonary embolism and non-embolic parenchymal lung disease but that these two groups differed significantly from normal. However, the overlap between the groups with lung disease and the normals was considerable which rendered the test of no clinical value.

Oestrogen-containing oral contraceptives are a known risk factor for the development of DVT and Duncan *et al.*[221] have studied the plasma βTG concentrations in a group of women using oral contraceptives and another group of smokers. They demonstrated elevated concentrations in smokers with even higher concentrations in those using oral contraceptives. However, patients who smoked and used oral contraceptives had the same concentrations as those who only smoked. A suggestion by the authors that the plasma βTG may be a useful screening test for at risk groups is contentious on two grounds. In the first case they were unable to show any correlation between the plasma βTG concentration and the oestrogen content of the oral contraceptive used. DVT is significantly commoner with the high oestrogen content preparations. Secondly, evidence from some of the major trials of DVT prophylaxis[132,175] suggests that smokers have a reduced incidence of deep vein thrombosis although they do have an increased risk of arterial thrombosis. It is interesting that in the study of Duncan *et al.*[221] smoking partly corrected the abnormality produced by the oral contraceptive.

Major infection seemed to be a potent cause of βTG release in our post-operation study although minor local sepsis seemed to have

no effect. van Hulsteijn *et al.*[243] studied 20 patients with bacterial infections and found that only 3 had persistently elevated plasma βTG concentrations. Rasi *et al.*[237] however showed that significantly elevated concentrations were found commonly in patients with septicaemia but less often in patients with pneumonia. They showed that the platelet count tends to rise after the first three days but were unable to correlate this closely with the βTG concentration. They comment, "Plasma βTG is not methodologically influenced by the platelet count." This supports the statement on page 51 of this thesis. In 5 patients with thrombocytopenia due to acute leukaemia they found low βTG concentrations, similar to the levels found in the patient with thrombocytopenia reported in this study.

A further study of the effect of prosthetic cardiac valves on plasma βTG has been reported by Pumphrey and Dawes[234] who showed a gradation in concentrations from the normal, through patients with heterografts and single mechanical valves, to those with double mechanical valves. They found that the plasma βTG was significantly higher in patients who embolised while on anticoagulants than in those who did not. Plasma βTG may therefore be a method of screening for patients at risk of embolism.

The effect of myocardial and coronary artery disease on plasma βTG remains contentious. Nichols *et al.*[231] in an angiographic study could not relate plasma βTG to the extent of angiographically proven coronary artery disease but did relate it to the extent of regional myocardial dysfunction. In contrast, Hughes *et al.*[228] in a study of

exercise induced myocardial ischaemia, failed to correlate the plasma βTG with the extent of post-exercise ischaemia on ECG but did relate it to the extent of coronary artery disease seen on angiography. In both these studies the correlation was not close (r = 0.53) but was significant.

In acute myocardial infarction Hughes *et al.*[227] showed that plasma βTG was elevated in only about half of the patients whereas a sixth of patients with non-coronary chest pain had elevated concentrations. However, Rasi *et al.*[236] using their own assay, found that 72% of patients with acute myocardial infarction, had elevated plasma βTG on the day of presentation. They felt that the plasma βTG was proportional to the extent of myocardial damage or stress to the body but make the telling comment, "The clinical usefulness of the test is not straightforward."

A study of the effect of exercise in patients with peripheral arterial disease was reported by Baele *et al.*[213] who showed a significant rise after 5 minutes of treadmill exercise. This rise may be indicative of increased platelet damage in a hyperdynamic, diseased peripheral circulation. If platelets are involved in the development of atherosclerosis as suggested,[28,49,204] increased platelet activity may result in a worsening of the disease process. However, there is no clinical evidence that exercise causes peripheral arterial disease to progress more rapidly.

Most people with stroke tend to have normal βTG concentrations[219,239] but Stewart *et al.*[239] did show that patients who had further "vascular events" had significantly elevated βTG concentrations.

These studies are all beset by the problems outlined in the section on arterial disease. All patients with arterial disease have diffuse disease which may have a variable effect on the plasma βTG outwith the effect of the disease in the area studied.

Several authors have reported on the effect of chronic renal failure on plasma βTG concentrations.[225,232,244] The plasma βTG tends to be elevated in relation to the degree of renal failure.[244] Both Guzzo *et al.*[225] and Petralito *et al.*[232] suggest that the rise is due to impaired renal handling of βTG rather than to increased platelet activity. If this is correct, the kidney must be an important site of βTG metabolism as the serum infusion study showed that only a tiny proportion of the infused βTG was excreted in the urine. Guzzo *et al.*[225] showed that in 3 patients with successful renal transplants the plasma βTG returned to normal.

Venous thrombosis is more common in patients with advanced malignancy and so Farrell *et al.*[223] assessed the platelet activity in patients with malignant disease by the plasma βTG. They showed that the patients with advanced disease had higher concentrations than those with early tumours. This may be due to platelet activation in an extensive abnormal tumour circulation. The patients in the surgical study in this thesis with malignant disease had plasma βTG concentrations slightly lower but not significantly different from those with benign disease. However, all these patients had early tumours and would be expected to have normal concentrations. Bidet *et al.*[214] report some interesting studies on the effect of chemotherapy. Despite maintenance of the platelet

count, the plasma βTG fell immediately after the first dose of chemotherapy. It rose only to fall again after the second dose. They raise the possibility of using the plasma βTG to assess the tumour burden and the effectiveness of chemotherapy.

Douglas *et al.*[220] also investigated patients before operation. They found that patients with operable malignancy had similar levels to those with benign disease but that both groups had significantly higher concentrations than the control group. The mean age of the control group was significantly lower than the two study groups. However, they showed that the correlation of age with the plasma βTG is not very close ($r = 0.33$). This lends support to the findings in this study of a raised plasma βTG on admission to hospital, possibly on the basis of stress. They also showed that infection resulted in a marked rise in plasma βTG.

In summary, much of the recent literature has produced findings in agreement with many of the studies in this thesis.

The further studies on the anticoagulant "cocktail" indicate that the Prostaglandin is probably beneficial but that other membrane active drugs such as Procaine have interesting potential. Aspirin appears to have little effect on plasma βTG in normal patients but may reduce spontaneous release from diseased platelets. Heparin also has little effect on short term βTG release. Most authors quote a poor accuracy in the diagnosis of venous thromboembolism using the plasma βTG which is in agreement with this study. The urinary βTG seems to be marginally better. The interesting suggestion that other body fluids such as saliva may yield

diagnostically useful βTG concentrations deserves further study.

The clinical value of βTG assessment in patients with arterial disease remains doubtful, presumably because of the extensive areas of potential platelet activation. Most studies are concerned with attempting to correlate plasma βTG with a small part of an extensive abnormal circulation. However, in patients with prosthetic heart valves, the plasma βTG may be of value in the detection of patients at risk of peripheral embolism.

Renal failure appears to increase plasma βTG, probably by reducing its metabolism by the kidney, and extensive malignancy is also associated with raised levels. An interesting potential use in the assessment of response to chemotherapy deserves further study.

These investigations further confirm the opinion expressed in this thesis that the assessment of βTG is of value in carefully controlled experimental situations but that its clinical value is limited by the many ways in which release may be stimulated *in vivo*.

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